ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons: http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons: http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license: https://creativecommons.org/licenses/?lang=en

Short- and medium-chain fatty acids as feed additives for the promotion of gut health in challenged weaning piglets

Memòria presentada per:

PAOLA LÓPEZ COLOM

Sota la direcció de les doctores:

SUSANA M. MARTÍN-ORÚE i LORENA CASTILLEJOS VELÁZQUEZ

Per accedir al grau de Doctor dins el programa de doctorat de Producció Animal del Departament de Ciència Animal i dels Aliments

Bellaterra, 2019

Susana M. Martín-Orúe i **Lorena Castillejos Velázquez**, investigadores del departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

certifiquen:

que la memòria titulada "**Short- and medium-chain fatty acids as feed additives for the promotion of gut health in challenged weaning piglets**", presentada per Paola López Colom per optar al grau de Doctor en Veterinària, ha sigut realitzada sota la seva direcció i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signen les presents a Bellaterra, 18 de febrer del 2019.

Dra. Susana M. Martín-Orúe   Dra. Lorena Castillejos Velázquez

La present memòria de tesi ha sigut realitzada gràcies a la concessió de la beca predoctoral FI-DGR 2016 per l'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) de la Generalitat de Catalunya i al finançament proporcionat pel Centro para el Desarrollo Tecnológico Industrial (CDTI) del Ministerio de Economía y Competitividad conjuntament amb Norel S.A provinent del projecte *PORCDIGEST* amb codi IDI-20140262.

Resum

El principal objectiu d'aquesta Tesi ha consistit en avaluar el potencial de dos noves formes d'àcids de cadena curta (AGCC) i mitjana (AGCM), combinades o no, com a suplement per a dietes de garrins deslletats a fi de reforçar-ne la salut intestinal i prevenir la invasió de patògens intestinals així com d'estudiar els possibles mecanismes d'acció dels additius.

Per tal d'assolir aquests objectius, es van dur a terme tres estudis de manera seqüencial. Al Capítol 4, es van avaluar dos formes protegides de butirat o heptanoat sòdics en garrins deslletats desafiats oralment amb *Escherichia coli* enterotoxigènica (ECET) F4. Al Capítol 5, es va avaluar una mescla de sals sòdiques d'AGCM provinents de destil·lats de coco davant de dos desafiaments orals amb patògens en garrins deslletats, concretament amb *Salmonella* Typhimurium i ECET F4. Al Capítol 6, es van avaluar les mateixes sals d'AGCM, però en aquest cas protegint el butirat o heptanoat sòdics, en garrins deslletats desafiats oralment contra ECET F4.

Es va seguir un protocol similar en tots els estudis. Breument, els garrins deslletats van ser transportats de granges comercials a les instal·lacions de la UAB. Cada grup tractat va constar de 8 corrals (rèpliques) amb tres garrins en cadascun. Després d'una setmana d'adaptació a les dietes i a les instal·lacions, es van desafiar els animals oralment amb el patogen corresponent i un animal per corral va ser eutanasiat a posteriori els dies 4 i 8 post-inoculació (PI). A tots els experiments, les variables mesurades consistien en avaluar el rendiment productiu, la resposta immunitària, la morfologia intestinal i la microbiota intestinal mitjançant la seqüenciació d'alt rendiment (SAR) del gen 16S de l'ARN ribosomal (ARNr).

Ambdós sals sòdiques de butirat o heptanoat (Capítol 4) van mostrar tenir impacte al llarg del tracte gastrointestinal (TGI) dels garrins tot i que la identificació dels analits corresponents, àcids butíric o heptanoic, no va evidenciar-ne l'increment de llurs concentracions al tracte inferior. A l'estómac, el butirat es va associar a una depressió de la fermentació làctica i l'heptanoat amb canvis al patró de fermentació. Ambdós additius van reduir els AGCC al còlon i es van incrementar les poblacions d'enterobacteris i coliforms a l'ili sense afectar la presència d'*E. coli* F4. A més, diferents poblacions del còlon es van veure modificades amb les sals àcides. Amb el butirat sòdic, va observar-se una pujada de *Prevotella* i una tendència cap a un menor ràtio Firmicutes:Bacteroidetes (F/B) (P = 0.11), mentre que l'heptanoat sòdic va incidir sobre algunes poblacions minoritàries, principalment de Clostridiales i Bacteroidales, i va promoure Actinobacteria.

Amb la mescla de sals sòdiques dels AGCM de destil·lats de coco (Capítol 5), tant *Salmonella* spp. com els enterobacteris van veure's reduïts consistentment a l'intestí posterior dels garrins després dels desafiaments amb *Salmonella* Typhimurium o ECET F4, respectivament. Tanmateix, depenent del patogen inoculat, els recomptes de limfòcits intraepitelials (LIE) van disminuir en els garrins suplementats desafiats amb *Salmonella*, però van augmentar després del desafiament amb ECET F4. Els additius també van modificar la microbiota del còlon. Després del desafiament amb *Salmonella*, la ràtio F/B va tendir a veure's reduïda (P = 0.13) mentre Fibrobacteres van créixer. Després del desafiament amb ECET F4, la suplementació d'AGCM va associar-se amb canvis en l'estructura global de la comunitat alhora que es va registrar l'augment de *Dialister* i també una tendènciae en la família Veillonellaceae (P = 0.14).

Finalment, la protecció tant de butirat com d'heptanoat sòdics amb la mescla de sals sòdiques d'AGCM de destil·lats de coco (Capítol 6) van donar lloc a respostes diferents en els garrins davant del desafiament amb ECET F4, amb què es van reflectir parcialment els efectes observats prèviament amb els compostos de manera individual. Per una banda, amb el butirat sòdic protegit amb AGCM no va ser capaç de reduir la col·lonització d'*E. coli* F4 però d'augmentar els recomptes d'enterobacteris i de cèl·lules cal·liciformes (a les vellositats) de l'ili i el TNF-α sèric. No obstant, amb aquest additiu també es va veure una increment de la ràtio F/B al còlon (P = 0.05). En quant a l'heptanoat sòdic protegit amb AGCM, amb la seva suplementació es van reduir la col·lonització d'*E. coli* F4 i Enterobacteriaceae, quantificada mitjançant la SAR del gen 16S de l'ARNr. Amb aquest additiu, els AGCC del còlon i les cèl·lules cal·liciformes de l'ili també van veure's reduïts.

Amb tot plegat, ambdós sals àcids protegides amb AGCM podrien estar reproduint, fins a cert punt, la suma dels efectes dels compostos individuals de cadascun dels additius. No obstant, els efectes observats dependrien del producte protegit. Mentre amb la protecció del butirat amb els AGCM no va poder-se prevenir la col·lonització de l'ECET F4, però sí incrementar la ràtio F/B com s'hauria observat prèviament amb els AGCM, amb la protecció de l'heptanoat amb els AGCM es va aconseguir controlar la presència del patogen, tal i com com s'havia observat prèviament amb els AGCM individualment amb efectes inhibitoris sobre la fermentació a còlon. Amb aquests resultats es podria suggerir una millor eficàcia de l'heptanoat sòdic protegit amb els AGCM de destil·lats de coco davant d'ECET F4, però no del butirat sòdic protegit. Per la seva banda, la forma protegida d'aquesta sal amb els AGCM prendria l'avantatge de la combinació dels efectes observats amb els AGCM sobre la microbiota i l'efecte tròfic del butirat en l'epiteli intestinal, cosa que ajudaria els animals a recuperar-se del dany causat pel patogen.

Resumen

El principal objetivo de esta Tesis ha consistido en evaluar el potencial de dos nuevas formas de ácidos grasos de cadena corta (AGCC) y media (AGCM), combinadas o no, como suplementos para dietas de lechones destetados a fin de reforzar su salud intestinal y prevenir la invasión de patógenos intestinales, así como estudiar los posibles mecanismos de acción de los aditivos.

Para conseguir tales objetivos, se llevaron a cabo tres estudios secuenciales. En el Capítulo 4, se evaluaron dos formas protegidas de butirato o heptanoato sódicos en lechones destetados desafiados oralmente con *Escherichia c*oli enterotoxigénica (ECET) F4. En el Capítulo 5, se evaluó una mezcla de sales sódicas de AGCM procedentes de destilados de coco frente a dos desafíos orales con patógenos en lechones destetados, concretamente con *Salmonella* Typhimurium y ECET F4. En el Capítulo 6, se evaluaron las mismas sales de AGCM, pero en este caso protegiendo el butirato o el heptanoato sódicos, en lechones destetados desafiados frente a ECET F4.

Se prosiguió con un protocolo similar en todos los estudios. Brevemente, los lechones destetados fueron transportados de granjas comerciales a las instalaciones de la UAB. Cada grupo tratado constó de 8 corrales (réplicas) con tres lechones en cada uno. Tras una semana de adaptación a las dietas y a las instalaciones, los animales fueron desafiados oralmente con el patógeno correspondiente y un animal por corral se eutanasió a posteriori los días 4 y 8 post-inoculación (PI). En todos los experimentos, las variables medidas incluyeron el rendimiento productivo, la respuesta inmunitaria, la morfología intestinal y la microbiota intestinal mediante la secuenciación de alto rendimiento (SAR) del gen 16S del ARN ribosomal (ARNr).

Ambas sales sódicas de butirato o heptanoato (Capítulo 4) mostraron tener impacto a lo largo del trato gastrointestinal (TGI) de los lechones si bien la identificación de los analitos correspondientes, ácidos butírico o heptanoico, no evidenció una mayor concentración de ninguno de ellos en el trato inferior. En estómago, el butirato se asoció con una depresión de la fermentación láctica y el heptanoato con cambios en el patrón fermentativo. Ambos redujeron los AGCC en colon y se incrementaron las poblaciones de enterobacterias y coliformes en íleon sin afectar *E. coli* F4. Diferentes poblaciones en colon se vieron modificadas con las sales ácidas. Con el butirato sódico, se observó una subida de *Prevotella* y una tendencia hacia una menor ratio de Firmicutes:Bacteroidetes (F/B) (P = 0.11), mientras que el heptanoato sódico incidió sobre algunas poblaciones minoritarias, principalmente de Clostridiales y Bacteroidales, y promovió Actinobacteria.

Con la mezcla de sales sódicas de los AGCM de destilados de coco (Capítulo 5), tanto *Salmonella* spp. como las enterobacterias se vieron reducidas consistentemente en el intestino posterior de

los lechones después de los desafíos con *Salmonella* Typhimurium o ECET F4, respectivamente. Asimismo, dependiendo del patógeno desafiado, los lechones respondieron de forma distinta a la suplementación. Mientras que los recuentos de linfocitos intraepiteliales (LIE) disminuyeron en los animales suplementados desafiados con *Salmonella,* estos aumentaron tras el desafío con ECET F4. Los aditivos también modificaron la microbiota del colon. Tras el desafío con *Salmonella*, la ratio F/B mostró una tendencia a verse reducida (P = 0.13) observándose también un incremento en Fibrobacteres. Bajo el desafío con ECET F4, la suplementación con AGCM se asoció con cambios en la estructura global del ecosistema y se registró un incremento en *Dialister* y también una tendencia en la familia Veillonellaceae (P = 0.14).

Finalmente, tanto la protección de butirato como de heptanoato sódicos con la mezcla de sales sódicas de AGCM de destilados de coco (Capítulo 6) dieron lugar a distintas respuestas en los lechones frente al desafío con ECET F4, reflejando parcialmente los efectos observados previamente con los compuestos de manera individual. Por un lado, el butirato sódico protegido con AGCM no fue capaz de reducir la colonización de *E. coli* F4 observándose un aumento en los recuentos de enterobacterias y de células caliciformes (en vellosidades) del íleon y el TNF-α sérico. No obstante, con este aditivo también se vio un incremento en la ratio de F/B en colon (P = 0.05). En cuanto al heptanoato sódico protegido con AGCM, su suplementación redujo la colonización intestinal por *E. coli* F4 y Enterobacteriaceae, cuantificada mediante SAR del gen 16S del ARNr. Con este aditivo, también se redujeron los AGCC en colon y los recuentos de células caliciformes en íleon.

Los resultados obtenidos con ambas sales ácidas protegidas con AGCM podrían estar reproduciendo, hasta cierto punto, la suma de los efectos individuales de cada uno de los aditivos. Los efectos observados dependieron, no obstante, de la mezcla protegida. Si bien la protección del butirato con los AGCM no pudo prevenir la colonización del intestino por ECET F4, pero sí incrementar la ratio F/B como se había visto con los AGCM, la protección del heptanoato con los AGCM sí consiguió controlar la presencia del patógeno, tal como se observó previamente con los AGCM individualmente con efectos inhibitorios sobre la fermentación colónica. Estos resultados sugieren una mayor eficacia del heptanoato sódico protegido con los AGCM de destilados de coco frente a ECET F4, pero no del butirato sódico protegido. Por su parte, la forma protegida de esta sal con los AGCM aunaría los efectos beneficiosos de los AGCM sobre la microbiota y el efecto trófico del butirato en el epitelio intestinal, lo que podría contribuir a una mejor recuperación de los animales tras el daño causado por el patógeno.

Summary

The main objective of this Thesis was to evaluate the potential of new forms of short- and medium-chain fatty acids either alone or in combination to be supplemented in the diets of weaning piglets to enhance their gut health and to control and prevent intestinal pathogens, as well as further study their possible mechanisms of action.

To accomplish these objectives, three studies were performed for a sequential response. In Chapter 4, two protected forms of sodium butyrate or sodium heptanoate were evaluated in weaned piglets orally challenged with enterotoxigenic *Escherichia coli* (ETEC) F4. In Chapter 5, the mixture of sodium salts of medium-chain fatty acids (MCFA) from coconut distillates was tested under two different pathogenic oral challenges in weaned piglets, *Salmonella* Typhimurium and ETEC F4. In Chapter 6, these MCFA sodium salts from coconut distillates protecting either sodium butyrate or sodium heptanoate were tested in weaned piglets orally challenged with ETEC F4.

A similar protocol was used in all the trials. Briefly, weaning piglets coming from commercial farms were transported to the experimental facilities of the UAB. Each treatment was tested in 8 pens (replicates) with three piglets each. After a week of adaptation to diets and facilities, animals were orally challenged with the corresponding pathogen and one animal per pen was euthanised on day 4 and 8 post-inoculation (PI). For all experiments, main variables assessed were animal performance, clinical signs, pathogen excretion, fermentation profile, immune response, intestinal morphology, and intestinal microbiota 16S rRNA gene high-throughput sequencing (HTS).

Both protected sodium salts of butyrate or heptanoate individually (Chapter 4) showed a broad impact along the gastrointestinal tract (GIT) of weaned piglets although traceability of butyric and heptanoic acids analytes did not evidence an increase of their concentration in the lower tract. In stomach, butyrate was associated to a depression of lactic fermentation and heptanoate with fermentation patterns being modified, while with both additives total short-chain fatty acids (SCFA) were reduced in colon and enterobacteria and coliforms increased in ileum without affecting *E. coli* F4 loads. Also, colonic microbial populations were modified with the acid salts, sodium butyrate was associated to an increase in the *Prevotella* group and trends for lower Firmicutes:Bacteroidetes (F/B) ratio ($P = 0.11$) while sodium heptanoate affected minor

populations, mainly from Clostridiales and Bacteroidales, as well as increased Actinobacteria.

With the mixture of sodium salts of coconut distilled MCFA (Chapter 5), *Salmonella* spp. and enterobacteria were seen to be consistently reduced in the hindgut of piglets after respective pathogen challenges, *Salmonella* Typhimurium or ETEC F4. Furthermore, piglets responded differently to the supplementation depending on the pathogen challenge being intraepithelial lymphocytes (IEL) reduced under the *Salmonella* challenge while increased under the ETEC F4 challenge. Also, the additive modified colonic microbiota, with F/B ratio tending to decrease (P = 0.13) and Fibrobacteres being enriched under the *Salmonella* challenge, and the drift of the microbiota communitie's structure concomitant with the rise of *Dialister* and trends on the same direction of Veillonellaceae (P = 0.14) under the ETEC F4 challenge.

Finally, protecting either sodium butyrate or sodium heptanoate with the mixture of sodium salts of coconut distilled MCFA (Chapter 6) promoted different responses against an ETEC F4 challenge in weaned piglets, partially reflecting the effects observed by the individual compounds. On one hand, MCFA-protected sodium butyrate was not able to reduce *E. coli* F4 colonisation but increased ileal enterobacteria, the numbers of goblet cells in ileum and serum TNF-α concentration. This additive increased, however, the F/B ratio in the colon (P = 0.05). Regarding MCFA-protected sodium heptanoate, *E. coli* F4 colonisation and Enterobacteriaceae were reduced, as observed in the 16S gene rRNA HTS. Also, with this additive, SCFA were reduced in colon, and goblet cells in ileum.

Altogether, both MCFA-protected acid salts may reproduce to some extent the sum of the individual compound effects although the results obtained with the functional protection differed depending on the products being combined. Whereas with MCFA protecting butyrate ETEC F4 colonisation was not prevented but F/B ratio was kept similarly to that observed with MCFA alone, the MCFA protecting form of heptanoate achieved to control the rise of the pathogen as MCFA salts did alone with depressing effect on colonic fermentation. These results would suggest an improvement in the efficacy against ETEC F4 of sodium heptanoate when is protected with MCFA from coconut distillates but not of sodium butyrate. For its part, protected form of sodium butyrate with MCFA would take advantage of combining positive effects seen with MCFA

on microbiota structure and the trophic effect of butyrate on intestinal epithelium that may help animals to better recover from the pathogen injury.

Index

Index of tables...... i

Index of figures...... ii

Abbreviations...... v

Chapter 1 General introduction...... 1

Chapter 2 Literature review...... 5

2.1 A brief contextualisation of weaning...... 7

2.1.1 Opportunistic enteropathogens...... 8

2.2 Generalities of organic acids...... 13

2.2.1 Growth enhancement...... 17

2.2.2 Effects on feed intake...... 21

2.2.3 Regulation of satiety and impact on metabolism...... 23

2.2.4 Motility and nutrient utilisation...... 25

2.2.5 Stomach barrier...... 27

2.2.6 Intestinal barrier...... 28

2.2.7 Inflammatory-immune equilibrium...... 30

2.2.8 Antimicrobial effects...... 31

2.3 Strategies to increase the efficacy of short- and medium-chain fatty acids.. 37

2.3.1 Microencapsulation...... 37

2.3.2 Derivatives with other chemical presentations...... 46

2.3.3 Blends and combinations with other additives...... 47

2.4 Microbiota 16S rRNA gene as a tool of assessment of in-feed additives activity...... 55

2.4.1 Changes on pig gut microbiota during first stages of life...... 57

2.4.2 Different microbial niches found along the GIT...... 62

2.4.3 Gut dysbiosis around weaning...... 67

2.4.4 Impact of antibiotics and their alternatives on GIT microbiota...... 70

Chapter 3 Objectives and experimental design...... 77

Chapter 4 Response of gastrointestinal fermentative activity and colonic microbiota to protected sodium butyrate and protected sodium heptanoate in weaned piglets challenged with ETEC F4........ 81

Chapter 5 Efficacy of salts of medium-chain fatty acids distilled from coconut oil against two enteric pathogens in weaned piglets........ 109

Chapter 6 Impact of sodium butyrate or sodium heptanoate protected with medium-chain fatty acids on the health of weaned piglets challenged with ETEC F4........ 139

Chapter 7 General discussion........ 169

7.1 Efficacy of protected sodium butyrate, protected sodium heptanoate, sodium salts of coconut MCFA or their combination on the control of intestinal bacterial infections........ 171

7.2 The reliability of the challenge model........ 179

7.2.1 Usefulness of the measured variables........ 182

7.3 Gut microbiota still to be deciphered........ 185

Chapter 8 Conclusions........ 193

Chapter 9 References........ 197

Appendix A........ 235

Appendix B........ 239

Index of tables

Table 2.1 Short- and medium-chain fatty acids trivial names and chemical formulas........ 13

Table 2.2 List of commercialised organic acids and blends and correspondent assigned function and use for animal target........ 15

Table 2.3 Presentation of all the works testing protected SCFA or MCFA compared classified based on the encapsulating agent........ 41

Table 2.4 Presentation of all the works testing of SCFA-MCFA-based blends with other organic acids and/or natural extracts, or lipase compared to the individual compound... 50

Table 2.5 Impact of different pathogen challenges inducing diarrhoeic processes on pig microbial populations evaluated through the HTS analysis of the 16S rRNA gene........ 69

Table 2.6 Impact of different in-feed antibiotics on GIT pig microbial populations evaluated through the HTS analysis of the 16S rRNA........ 71

Table 2.7 Impact of different antibiotic alternatives (probiotics and organic acids) on pig GIT microbial populations evaluated through the HTS analysis of the 16S rRNA........ 74

Table 4.1 Ingredient composition and nutrient analysis of the basal diet as-fed basis (g/kg)........ 86

Table 4.2 Effect of the experimental treatments on growth performance........ 92

Table 4.3 Effect of experimental treatments on ileal histomorphometry on days 4 and 8 PI........ 93

Table 4.4 Effect of the experimental treatments on enterobacteria and coliform plate counts and pathogenic *E. coli* F4 on days 4 and 8 PI........ 98

Table 5.1 Ingredient composition (g/kg) and chemical analysis (%) of the basal diet as-fed basis........ 114

Table 5.2 Effect of the experimental treatments on growth performance in *Salmonella* and ETEC F4 trials........ 121

Table 5.3 Effect of the experimental treatments on the number of animals with countable numbers of *Salmonella* spp. (>10^3 log CFU/g FM) in different post-inoculation (PI) days in the *Salmonella* trial........ 123

Table 5.4 Effect of the experimental treatments on microbial plate counts on days 4 and 8 PI in the ETEC F4 trial........ 125

Table 5.5 Effects of experimental treatments on intestinal fermentation on days 4 and 8 PI in *Salmonella* and ETEC F4 trials........ 131

Table 5.6 Effect of experimental treatments on ileal histomorphometry on days 4 and 8 PI in *Salmonella* and ETEC F4 trials........ 132

Table 5.7 Pearson's correlations (*r*) between IEL or valeric acid and bacterial populations (*r* ≥0.5) for *Salmonella* and ETEC F4 trials........ 137

Table 6.1 Ingredient composition and nutrient analysis of the basal diet as-fed basis (g/kg)........ 144

Table 6.2 Effect of experimental treatments on animal performance in Trial 1 and Trial 2........ 150
Table 6.3 Effect of experimental treatments on serum inflammatory response on days 4 and 8 PI in Trial 1 and Trial 2........ 151
Table 6.4 Effect of experimental treatments on ileal histomorphometry on days 4 and 8 PI in Trial 1 and Trial 2........ 152
Table 6.5 Effect of experimental treatments on ileum and colon fermentation on days 4 and 8 PI in Trial 1 and Trial 2........ 153
Table 6.6 Effect of experimental treatments on microbial plate counts on days 4 and 8 PI in Trial 1 and Trial 2........ 155
Table 6.7 Effect of experimental treatments on pathogenic *E. coli* F4 on days 4 and 8 PI in Trial 1 and Trial 2........ 155
Table 6.8 Effect of experimental treatments on the relative abundance of families and genera on day 4 PI in Trial 1 and Trial 2........ 159
Table 7.1 Inflammatory response and diarrhoea and rectal temperature outcomes obtained in the three different ETEC F4 trials and *Salmonella* Typhimurium trial........ 180
Table 7.2 Correlations (Spearman correlation, ρ_S) between intestinal variables (from ileum digesta and mucosa scrapes or colon digesta) and growth and clinical performances among trials during the infectious period........ 183
Table 7.3 Microbial groups significantly (adjusted *P* value <0.10) modified by the experimental additives from Chapters 4, 5, and 6........ 191

Index of figures

Figure 2.1 Pathogenesis of enterotoxigenic *Escherichia coli* (ETEC) F4................................ 10
Figure 2.2. Pathogenesis of *Salmonella*.. 10
Figure 2.3 Network representing the most relevant published reviews of organic acids (related to the host physiology and as feed additive strategy to replace antibiotic use) citing each other.. 14
Figure 2.4 Average daily gain (ADG) differential between treated group and the correspondent control group among 62 published papers.. 17
Figure 2.5 Gain-to-feed ratio (GF) differential between treated group and the correspondent control group among 51 published papers.. 18
Figure 2.6 Average daily feed intake (ADFI) differential between treated group and the correspondent control group among 49 published papers.. 22
Figure 2.7 Disruption of the cell membrane, vacuoles, and disorganisation of the cytoplasm of *Clostridium difficile* with lauric acid treatment (A). Leakage and subsided of the cell membrane of *Staphylococcus aureus* with lauric acid treatment (B). Cytosol disorganisation of *Clostridium perfringens* with lauric acid treatment (C).......................... 32
Figure 2.8 *E. coli* (log_{10} CFU/g FM) differential between treated group and the correspondent control group among 21 published papers.. 34
Figure 2.9 Simple flowchart of the spray drying process.. 39
Figure 2.10 Main microbe-to-host and host-to-microbe interactions among gut microbiota and pig (host) depicted in literature.. 55
Figure 2.11 16S rRNA 2D structure with each one of the nine hypervariable regions differently coloured.. 57
Figure 2.12 Enterotype-like clusters in adult humans from different nationalities consisting of *Prevotella*, *Ruminococcus*, and *Bacteroides*, and in large white piglets across five age strata (14, 26, 60, and 70 days old) consisting of *Prevotella* and Ruminococcaceae.. 59
Figure 2.13 Evolution of the relative abundant (%) of the main phyla described over pig's life with the analysis of faecal samples.. 61
Figure 2.14 Relative abundance (%) of the main phyla found along the luminal GIT segments of the pig.. 63
Figure 2.15 Relative abundance (%) of the main phyla found along the mucosa of each GIT segment in the pig.. 66
Figure 4.1 Evolution of mean faecal scores in the different experimental groups CTR, BUT, and HPT along the post-inoculation (PI) period.. 93
Figure 4.2 Percentage of piglets with detectable levels of each organic acid in stomach in the different experimental groups CTR, BUT, and HPT.. 95
Figure 4.3 Lactic acid and total SCFA along the gastrointestinal tract in the different experimental groups CTR, BUT, and HPT.. 96
Figure 4.4 Mean relative abundance (%) of top 10: phyla; families; and genera, in the different experimental groups.. 99

Figure 4.5 Differentially abundant taxa (ln change and FDR-adjusted *P* <0.15) between: (A) BUT vs. CTR; and (B) HPT vs. CTR........ 101
Figure 5.1 Evolution of faecal scores along the post-inoculation (PI) period in *Salmonella* and ETEC F4 trials by experimental group CTR, and DIC........ 122
Figure 5.2 Number of animals with different range levels of *Salmonella* spp. counts in faecal samples at days 1, 3 and 7 PI and caecal content at days 4 and 8 PI (*Salmonella* trial)........ 124
Figure 5.3 Non-metric dimensional scaling (NMDS) plot of dissimilarity matrix based on Bray-curtis distance clustered by experimental diets CTR, and DIC for each challenge..... 126
Figure 5.4 Ln changes promoted by DIC supplementation (adjusted *P* <0.20) in taxa under *Salmonella* challenge or ETEC F4 challenge........ 128
Figure 5.5 Heatmap of differential abundant OTUs (relative abundance and adjusted *P* value <0.05) between DIC and CTR groups in each pathogen challenge: *Salmonella* trial; and ETEC F4 trial........ 129
Figure 6.1 Individual rarefaction curves (calculated richness) from day 4 PI corresponding to CTR animals and animals treated with BUT+ in Trial 1 or treated with HPT+ in Trial 2........ 156
Figure 6.2 Dendrogram of UPGMA hierarchical clustering (average method) based on Bray-curtis distances from day 4 PI representing individual animals treated CTR diet or supplemented with BUT+ in Trial 1 or with HPT+ in Trial 2........ 157
Figure 6.3 Relative abundances (%) of phyla represented as the average for CTR groups and animals treated with BUT+ in Trial 1 or treated with HPT+ in Trial 2 on day 4 PI........ 158
Figure 6.4 Heatmap of the effects of experimental additives BUT+ in Trial 1, and HPT+ in Trial 2 on the relative abundance of OTUs (%) change against CTR (adjusted-FDR *P* value <0.05)........ 160
Figure 7.1 Average daily feed intake (ADFI) along the experimental period for each one of the additives tested and their respective control from Chapters 4 (CTR 1, ETEC trial), 5 (CTR 3 & 4, ETEC & *Salmonella* trials), and 6 (CTR 2 & 3, ETEC trials)........ 174
Figure 7.2 Coliform counts quantified in colon digesta and ileum mucosa scrapes in the three different ETEC trials included in Chapters 4 (ETEC 1 Trial), 5 (ETEC Trial 3 and *Salmonella* Trial), and 6 (ETEC Trials 2 & 3)........ 181
Figure 7.3 *E. coli* F4 gene copies quantified in colon digesta and ileum mucosa scrapes in the three different ETEC trials included in Chapters 4 (ETEC 1 Trial), 5 (ETEC Trial 3 and *Salmonella* Trial), and 6 (ETEC Trials 2 & 3)........ 181
Figure 7.4 Microbiota profiles of phyla composition in healthy weaned piglets and pathogen-challenged piglets compiled from the literature, and from the results of the 16S rRNA sequencing of the colonic microbiota from Chapters 4 (ETEC Trial 1), 5 (ETEC Trial 3 & *Salmonella* Trial), and 6 (ETEC Trial 2 & 3) are presented........ 187
Figure 7.5 Non-metric dimensional scaling (NMDS) of the constraint disposition of the average microbial populations structure among the five studies (corresponding to a total of four trials) within Chapters 4 (ETEC Trial 1), 5 (*Salmonella* Trial & ETEC Trial 3) and 6 (ETEC Trials 2 & 3)........ 189

Abbreviations

ABC	Acid-binding capacity
ADFI	Average daily feed intake
ADG	Average daily gain
AMR	Antimicrobial resistance
ANOSIM	Analysis of similarities
BHI	Brain heart infusion
BW	Body weight
CD	Crypt depth
CEAAH	Ethical committee on human and animal experimentation
CFA	Colonisation-factor antigens
CFU	Colony forming units
CLDN	Claudin gene
DM	Dry matter
ETEC	Enterotoxigenic *Escherichia coli*
FA	Fatty acid
FAS	Fatty acid synthase
FDR	False discovery rate
FM	Fresh matter
GF	Gain-to-feed ratio
GC	Goblet cells
GI	Gastrointestinal
GIT	Gastrointestinal tract
HDAC	Histone deacetylase
HTS	High-throughput sequencing
IL	Interleukin
IPEC	Intestinal porcine epithelial cells
LAB	Lactic acid bacteria
LPS	Lipopolysaccharides
LA	Luria agar
Pig-MAP	Pig-major acute-phase protein
MCFA	Medium-chain fatty acid
MCT	Medium-chain fatty triglyceride
MCT1	Monocarboxylate transporter 1 gene
MIC	Minimum inhibitory concentration
NMDS	Non-metric dimensional scaling
OA	Organic acid
OCLN	Occludin gene
OTU	Operational taxonomic unit
PI	Post-inoculation
PPAR	Peroxisome proliferator-activated receptor
PYY	Peptide Y
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
SCFA	Short-chain fatty acid
SCOA	Short-chain organic acid
TNF-α	Tumour-necrosis factor-α
VFA	Volatile fatty acid

Chapter 1 General introduction

Antibiotics have been an important part in pig production and have aided to achieve the current productive levels. Still often, the weaning is a delicate period to manage. Piglets are prematurely separated from their mothers and given new solid feed, mixed with other unfamiliar mates and confronted with an undeveloped immune and gut microbial ecosystem (Jensen and Recén, 1989; Lallès et al., 2007; Min and Rhee, 2015). This scenario may predispose or protect pigs from intestinal pathogens, for instance, enterotoxigenic *Escherichia coli* (**ETEC**) F4 as the agent to cause the post-weaning diarrhoea (Dou et al., 2017), or *Salmonella* spp. (Stecher et al., 2010), causing later the growth impairment of the animals and economic losses to the farm, as well as of public concern as a common foodborne pathogen. To deal with these problems, farmers resorted antibiotics yet widely used prophylactically in animal farming to maintain potential pathogens far from the animals, and now, Europe is caught up with the concerns about antimicrobial resistance (**AMR**) rise (EFSA, 2018), partly because of this.

Turning down a practice fully stablished becomes troublesome. Removal (or reduction) of antibiotics implies a rethinking of the way the meat is produced. Combined management strategies as well as more environmental-friendly alternatives to benefit animals in terms of (gut) health and welfare are necessary. Nonetheless, undoubtedly, alternative dietary strategies such as additives are a target to be developed, as one of the five purposed objectives included in the international plan of the WHO (2015), to invest in new medicines and alternative interventions to antibiotics. In this line, several strategies have been gaining support along the years trying to cope with this scenario. In livestock, feed additives have been designed with the aim to replace, or at least, minimise the use of antibiotics, by resembling the activity of these.

Organic acids have fitted in well with this goal as antibiotic substitutes since they have shown potential as growth promoters, often linked to the modulation of the microbiota and the prevention of pathogen colonisation, the boosting of the immune system and the promotion of the digestive function in piglets (Mroz, 2005). Formerly, the so-called “classical” organic acids, e.g. lactic, citric, formic, or acetic acids, were seen capable to control intestinal populations and enhance the performance of the animals (Cole et al., 1968; Edmonds et al., 1985), implementing the technological application as feed preservers because of their acidifying capability (Wilson & Wilkins, 1973; Woolford,

1975). In the same vein, short-chain fatty acids (SCFA), naturally produced in the gastrointestinal tract (GIT) through the microbial fermentation, were early seen to have also antibacterial properties (Prohászka, 1980). In this sense, SCFA gained interest since they occur naturally and are related to gut health (Prohászka, 1980; Ríos-Covián et al., 2016). In the same line, medium-chain fatty acids (MCFA) attained again further attention because of the demonstrated antimicrobial effect for long time (Sheu and Freese, 1972; Desbois and Smith, 2010), as well as they are commonly found in natural sources like enriched-vegetable oils (Decuypere & Dierick, 2003), what at the moment represents no negative ecological impact nor associated AMR.

A literature compilation of the studies that tested the products based on these monocarboxylic acids, SCFA and MCFA, will be presented in this PhD dissertation to summarise which have been the main results and trying to elucidate which is the fundament sustaining their efficacy, or not. Also, this PhD dissertation will review the evolving feed technology applied to improve the efficacy and delivery of these compounds and finally present and discuss the evaluation of new forms of sodium salts of SCFA and MCFA under two different pathogen challenges, ETEC F4 or *Salmonella enterica* serovar Typhimurium, in weaning piglets. The results obtained will bear further testimony of their potential and help better understanding their mechanisms of action around the gut health and pathogen control, especially focused on the study of the hindgut microbiota ecosystem as an “organ” target of the entire animal health.

Chapter 2 Literature review

2.1 A brief contextualisation of weaning

Weaning is a long-standing problem in the pig production systems. As introduced, the current weaning is a challenging moment for the piglet associated to multiple stresses all at once. The piglet has to cope suddenly with changes in terms of social behaviour, nutrition, organic development and functional and environmental challenges. Weaning takes place at an early age between 3 and 4 weeks of age, while in nature systems occurs gradually around 2-3 months of age (Michiels et al., 2013; Moeser et al., 2017; Salazar et al., 2018).

Two phases have been described after weaning. Initially, an acute phase from 0 to 5 days post-weaning takes place, more related to fasting rather than the diet change. In this moment, brush border enzymes are less, villi are impaired already after 24 h and intestinal permeability increased, mainly in the proximal parts, where digesta is first arriving and nutrient degradation and absorption occurs (Hampson, 1986; Montagne et al., 2007; Liu, 2015). Furthermore, the intestine atrophy is accompanied by an immediate over-expression of inflammatory cytokines along the intestine of the piglets (Pié et al., 2004) and also at systemic inflammatory response (Piñeiro et al., 2009). Moreover, the loss of maternal antibodies and the mucosal immune system compromised after weaning and less reactive to foreign antigens (Bailey et al., 2005) need support. The second phase of adaptation encloses from 5 to 15 days post-weaning, associated to the recovery of the impairment of the villi and with changes in microbiota and distal parts of the gut (Hampson, 1986; Pluske et al., 2003; Montagne et al., 2007).

In this regard, microbiota is an active part of the intestine as a big place of defence of the host in a close relationship with the immune system. Intestinal microbiota is also readapted to the new milieu that is transient from starvation to new solid diets resulting in unstable populations that have to become complex and stable in the adult pig. In this sense, its development will be accompanied by its increasing fermentative activity producing volatile fatty acids (VFA), which have shown to be protective (Heo et al., 2013).

Within this context, introduction of new feed strategies to the weaning transition to skip the use of antibiotics and maintain the intestinal pathogens far from young piglets requires a more comprehensive evaluation of the health status of the animals. Human

medicine has recategorised the concept of "gut health" (Wilmore, 1997; Coussement, 2002) migrating to animal production as synonym of animal health. The animal performance has been indeed associated to a healthy gastrointestinal tract (GIT) in terms of nutrient digestion and absorption, host metabolism and energy, mucus barrier and mucosal immune functions, and microbiota profiling (Kogut & Arsenault, 2016).

2.1.1 Opportunistic enteropathogens

During weaning, this entire dysfunctional picture of piglet's intestine (inflammatory response, intestinal digestion and barrier functions, and microbiota ecosystem) can be largely amplified by enteric pathogens, such as pathogenic *Escherichia coli* or *Salmonella* spp. These are closely related genera members of Enterobacteriaceae, gram-negative, non-spore-forming and rod-shaped bacteria, facultative anaerobes and motile (Loynachan, 2005; Fairbrother & Gyles, 2012). These two bacteria can defeat the immunity of the host and have shown acid-resistance mechanisms developed to surpass the gastric barrier (Foster, 2004; Fàbrega & Vila, 2013). Moreover, these opportunistic pathogens encounter high possibilities to colonise the gut of weaning piglets considering that at this age the passive immunity of the colostrum starts to fail, there are a weaker stomach acidity, and an unstable intestinal microbiota.

E. coli is ubiquitous and a normal commensal of the tract of mammals and birds. Among the wide classification of serotypes, a small proportion are pathogenic. The serotypes of *E. coli* can be classified depending on the surface antigen, the somatic antigen (O) and flagellar antigen (H)[1]. The O antigen is linked to the core lipopolysaccharide (LPS) buried beneath the O, common to all gram-negative bacteria (Evans & Evans, 1996). It is a heat-stable polysaccharide present in the outer surface, and O specificity differs depending on the amino sugar composition and the sequence of the polysaccharide chains composed of 6-5 sugars (D'Aoust & Maurer, 2007). The antigen H is carried in the flagellum that provides the motive power to the bacterium (Wang et al., 2003).

[1] At the very beginning, the colonies of intestinal bacilli growing in agar and forming a thin halo along the agar received the name *Hauch* (H), meaning breath in German. Thus, they were motile isolates, while those from immotile bacteria or without flagellum, more compact without a halo, were called *ohne Hauch* (O), without breath, being translated afterwards to flagellar and somatic, respectively (Lutwick, 2014).

Enterotoxigenic *E. coli* (ETEC) is one of the pathotypes[2] of *E. coli* characterised by adhering to intestinal epithelium and colonise the mucosal surface. This pathotype possesses fimbrial adhesins (F) known as colonisation-factor antigens (CFA). These are unrelated to O nor H, and common pili and much thinner and rigid than flagella (Evans & Evans, 1996; Zhou et al., 2014). The most common fimbrial types of *E. coli* causing post-weaning diarrhoea in piglets are F4 or F18, depending on the serological reactivity. The most prevalent type is F4, often referred simply to F4 (previously related to capsular -K-antigen, K88), along with the antigenic subtypes F4ac and most of the isolates being O149 (Schroyen et al., 2012).

The pathogen is introduced and transmitted via faeco-oral and can be maintained along different batches (Figure 2.1) (Fairbrother et al., 2005). ETEC adheres to the surface receptors of mainly jejunum and ileum mucosa, almost with no alterations of the microvilli cytoskeleton (Sansonetti, 2002). Thereafter, ETEC produces two major enterotoxins, thermo-stable STa and STb, and thermo-labile enterotoxin LT, which act as antagonists of sodium/water reabsorption and agonists of chlorine/water secretion and trigger the loss of electrolytes and water (Fairbrother & Gyles, 2012), and the rapid onset of a non-bloody watery liquid diarrhoea. The clinical disease can be accompanied either with no fever or even with loss of body temperature (Spitzer et al., 2014; Rhouma et al., 2017), and animals show dehydration and emaciation and mortality up to 30 % (Fairbrother & Gyles, 2012; Rhouma et al., 2017). The enterotoxin genes usually are carried out with the fimbrial gene in the same plasmid, therefore most of the cases of ETEC diarrhoea are caused by combining CFA and enterotoxins (Evans & Evans, 1996). Nonetheless, ETEC can also colonise the pig without obvious symptoms. Moredo et al. (2015) detected up to 66 % of weaning piglets carrying the pathogen ETEC subclinically.

[2] Variant classified based on differences on virulence phenotype that characterise the pathology (Sturhan, 1985).

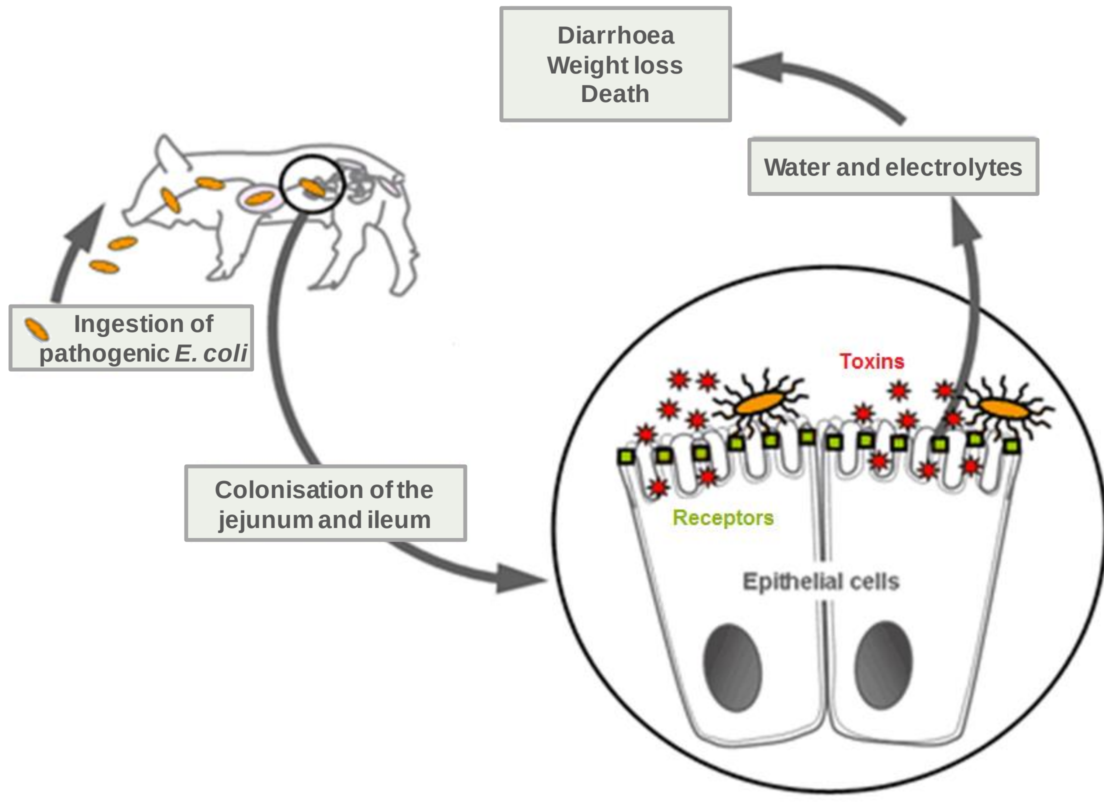


Figure 2.1 Pathogenesis of enterotoxigenic *Escherichia coli* (ETEC) F4 (from EcL, 2004).

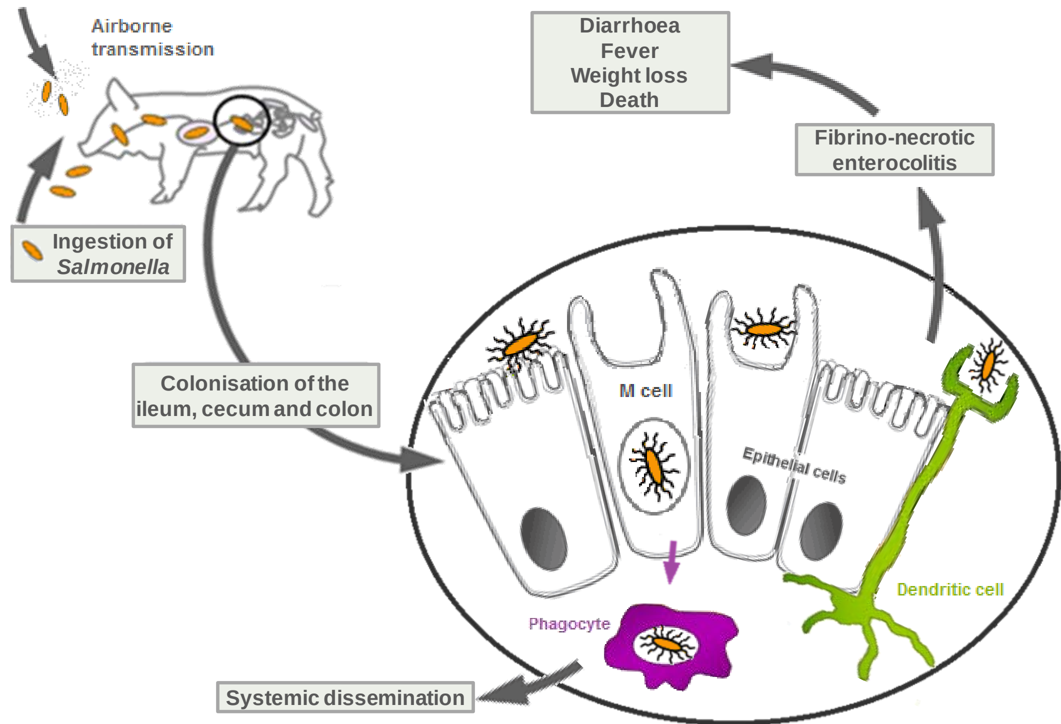


Figure 2.2 Pathogenesis of *Salmonella* (adapted from Sansonetti, 2002, and Fàbrega & Vila, 2013).

Regarding *Salmonella* spp., this enteropathogen is also ubiquitous and commonly found in the tract of animals. The two species in the genus *Salmonella* are *Salmonella enterica* and *Salmonella bongori*. One of the most concerning serovars among *Salmonella enterica* subsp. *enterica* is the non-host adapted *S. enterica* serovar Typhimurium as a foodborne pathogen beyond the clinical disease affecting pigs (Loynachan, 2005). More precisely, *Salmonella* Typhimurium is classified by the serovar antigenic formula of White-Kauffman-le-minor scheme as 1,4,[5],12:i:1,2 related to somatic (O) and flagellar antigens (H, phase 1 and if present, phase 2). As pathogenic *E. coli*, *Salmonella* serovars can be motile and present flagellar (H), somatic (O) and capsular (K) antigens (Grimont & Weill, 2007). The full serovar name is only assigned to isolates of *Salmonella enterica* subsp. *enterica*, which meet the full antigenic definition for a serovar. For example, *Salmonella enterica* subsp. *enterica* 4,5,12:i:- is a commonly encountered, monophasic variant of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Ryan et al., 2017) lacking the phase-2 flagellar antigen.

Salmonella is transmitted as ETEC, via faeco-oral between pigs, despite it has also been reported to be transmitted via airborne (Figure 2.2). Tonsils are the first site of colonisation in pigs. *Salmonella* colonises preferentially the ileum, caecum and colon probably to surpass the bile salts and other digestive compounds of the small intestine (Boyen et al., 2008a). *Salmonella* is an entero-invasive bacterium that internalises to the intestinal epithelial cells by a "trigger process"[3] with the contact with M cells, what induces massive cytoskeleton changes that procure a ruffling event whereby the bacterium gets into a macropinocytic vacuole to get inside the associated lymphoid tissue beneath the intestinal mucosa (Sansonetti, 2002), hence, the predominant entrance are Peyer's patches (Carlson et al., 2012). This process mainly encompasses the expression and production of several pro-inflammatory cytokines such as tumour-necrosis factor (TNF-α) and interleukin-8 (IL-8), thus recruits neutrophils, and macrophages that phagocyte *Salmonella* (Sansonetti, 2002; Fàbrega & Vila, 2013). Also, dendritic cells can directly take up *Salmonella* from the intestinal lumen through the tight junctions

[3] Process whereby bacteria induce massive cytoskeletal changes in the mammalian cell in the site of contact, thereby causing a ruffling process that internalises the bacterial body in a macropinocytic vacuole (Sansonetti, 2002).

(Sansonetti, 2002; Fàbrega & Vila, 2013). Once entrapped, *Salmonella* can disseminate systemically via lymph or bloodstream and replicate preferentially in liver and spleen, although not persistently (Boyen et al., 2009).

The main lesion is a severe fibrino-necrotic enterocolitis and the lymph nodes can be enlarged and oedematous. Pigs present clinical signs of diarrhoea and fever, anorexia and dehydration, what further compromises their growth (Loynachan, 2005). This pathology demands high amounts of nutrients to support the immune system, but it is a self-limiting disease, and animals can remain as carriers of the pathogen (Carlson et al., 2012). In the field conditions, young pigs can carry latent infections and shed *Salmonella* intermittently along their lives (Loynachan, 2005), faecal prevalence ranging from 30 to 90 % in subclinical-course infections in nursery units (Kranker et al., 2003; Vigo et al., 2009). This fact may unleash the underestimation of their presence (Rasschaert et al., 2016) when pretending to fight against the pathogen.

2.2 Generalities of organic acids

Traditionally, the organic acids (OA) receiving most of the attention have been short-chain organic acids (SCOA), because of their suitability as food and feed preservatives, as well as their specific antimicrobial activity associated to performance benefits in animals. SCOA consist of a general structure of R-COOH either conformed of simple monocarboxylic acids such as formic, acetic, propionic, and butyric, or bearing a hydroxyl group usually in the α-carbon such as lactic, malic, tartaric or citric acids, as well as other acids with double bonds such as sorbic or fumaric acids (Dibner & Buttin, 2002).

Monocarboxylic acids (fatty acids, FA) are contained in natural feed resources and are versatile in terms of physicochemical properties and effects on the intestinal and post-absorptive function of the animals (Table 2.1). Especially the short-chain monocarboxylic acids, i.e. short-chain fatty acids (SCFA), have been deeply studied in their role on the hindgut fermentation in non-ruminants (Mroz et al., 2006). Most recently, medium-chain fatty acids (MCFA) have gained importance due to their antibacterial effects (Desbois & Smith, 2010; Zentek et al., 2011; Baltić et al., 2017; van der Aar et al., 2017). They are saturated and unbranched-fatty acids of between 6 and 12 carbon-chain lengths, contained in milk fat as triglycerides (medium-chain triglycerides, MCT) or in natural sources such are coconut or palm kernel (Dierick et al., 2003; Marten et al., 2006; Thompson et al., 2007).

Table 2.1 Short- and medium-chain fatty acids trivial names and chemical formulas.

Cx	Trivial name	Chemical formula
C1	Formic	$HCOOH$
C2	Acetic	CH_3COOH
C3	Propionic	CH_3CH_2COOH
C4	Butyric	$CH_3CH_2CH_2COOH$
C5	Valeric	$CH_3CH_2CH_2CH_2COOH$
C6	Caproic	$CH_3CH_2CH_2CH_2CH_2COOH$
C7	Enanthic	$CH_3CH_2CH_2CH_2CH_2CH_2COOH$
C8	Caprylic	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2COOH$
C9	Pelargonic	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$
C10	Capric	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$
C11	Undecylic	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$
C12	Lauric	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$

Actually, the better evidence is the wide number of products based on these compounds already commercialised (Table 2.2) and focused on improving the acidification of stomach at early ages, the modulatory effect on gut microbiota and the reinforcement of the gut barrier.

With especial mention to SCFA and MCFA, organic acids are still a hot research area with a great number of reviews published over the past 30 years (Figure 2.3). The compiled results showed evidences, as well as weaknesses, of SCFA and MCFA to promote the gut health and control potential pathogens, mainly focused in the weaning period. Their actions are mostly based on their capability to improve the acidifying function of stomach, support the digestion of nutrients, and inhibit growth of pathogens and their ability to modulate intestinal microbiota. In the following sections there will be reviewed results of SCFA and MCFA on growth and health status based on all these possible modes of action.

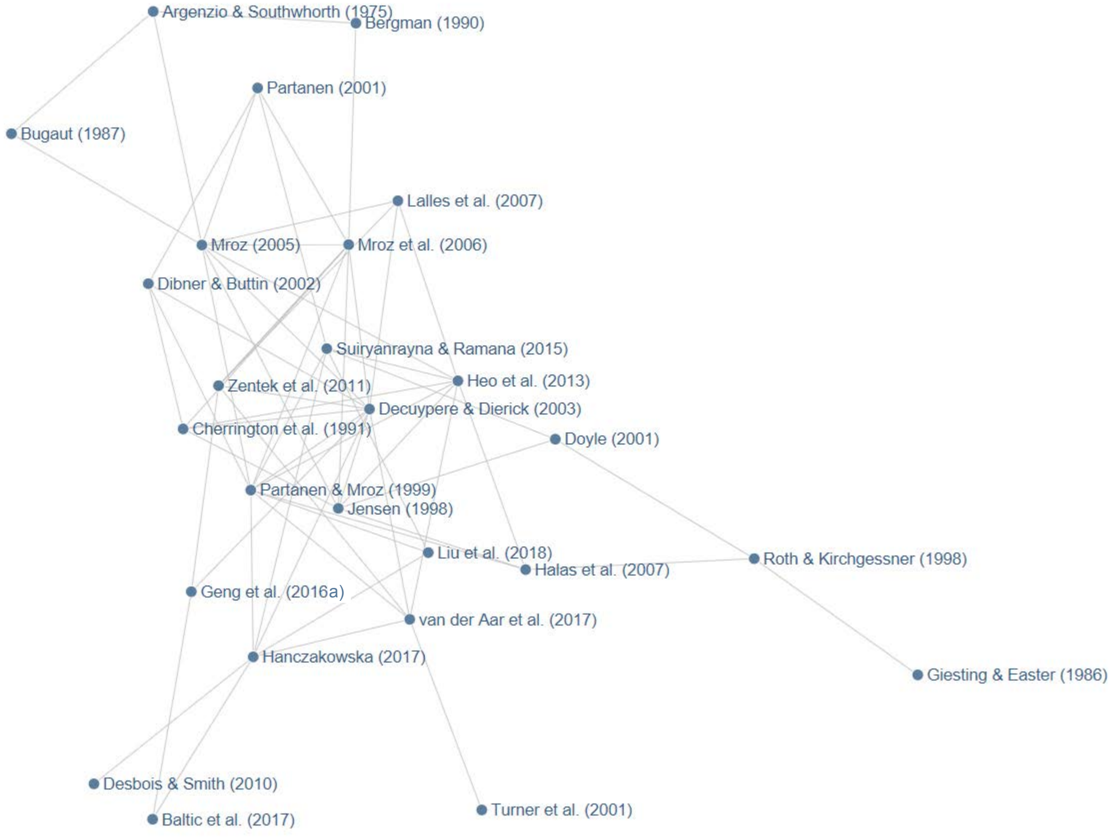


Figure 2.3 Network representing the most relevant published reviews of organic acids (related to the host physiology and as feed additive strategy to replace antibiotic use) citing each other. The network graph was performed with simpleNetwork() function from the package networkD3 (Allaire et al., 2017).

Table 2.2 List of commercialised organic acids and blends and correspondent assigned function and use for animal target (adapted from nutriNews (2018)).

	Action	Composition	Animal specie	Technology
Acidifier	acidifier and gut microbiota modulator	lactic, acetic, formic, propionic, butyric, propylene glycol	swine, poultry, rabbit	
	acidifier for water and feed	formic, orthophosphoric, propionic, NH_3-formate, NH_3-propionate	multispecies	
	acidifier	phosphoric, citric, fumaric	multispecies, especially for piglets, poultry, calves, rabbits	
	acidifier	fumaric, lactic, formic, propionic, citric	multispecies	
	acidifier	fumaric, malic, phosphoric, citric	piglets, poultry	microencapsulated
	acidifier and antibacterial	formic, lactic, essential oils	swine, poultry	
	acidifier and electrolytic balance	sodium formate	swine, poultry, rabbit	powder
	gut acidifier	formic, lactic, sorbic, acetic	swine	free acids
	acidifier for water	formic, propionic, acetic	monogastrics	
	gut acidifier	copper, formic, acetic, propionic, NH_3-formate		liquid
	acidifier and gut microbiota modulator	orthophosphoric, fumaric, citric, propionic, lactic, acetic	monogastrics	powder
	acidifier and antibacterial	citric, MCFA, fumaric, formic, essential oil microencapsulated		microencapsulated
	acidifier and antibacterial	formic, acetic, NH_3-formate, L-ascorbic, citric, copper, zinc	swine, poultry	free and buffered acids
	antibacterial	formic, acetic, NH_3-formate, propionic, glyceril-polyethylene glycol ricinoleate, coconut and palm oil distillates	poultry, swine, rabbits	free and buffered SCFA with MCFA
	acidifier and antibuffering	sorbic, formic, acetic, lactic, propionic, NH_3-formate, citric	swine	
	acidifier, hygieniser	MCFA, lactic, formic, propionic, monobutyrin	swine, poultry	synergy
Gut microbiota	acidifier and gut microbiota modulator	SCFA, MCFA, organic acid salts, essential oils	multispecies, especially monogastrics	
	acidifier and gut microbiota modulator	SCFA encapsulated, MCFA encapsulated, organic acid salts, mannan-oligosaccharides (MOS), essential oils	multispecies, especially monogastrics	
	gut microbiota modulator	esterified MCFA	monogastrics	esters
	gut epithelial integrity	butyric triglyceride, butyric monoglyceride	monogastrics	esters
	gut microbiota modulator	lauric monoglyceride, esterified SCFA, esterified MCFA	monogastrics	esters
	antibacterial and gut health	SCFA monoglycerides, SCFA diglycerides, MCFA monoglycerides, MCFA diglycerides	multispecies, especially piglets, poultry, fish, rabbits	esters

Table 2.2 (continued).

Gut microbiota modulation	antibacterial	organic acids, essential oils		microencapsulated
	antibacterial	calcium formate, citric	multispecies	encapsulated
	nutrient use and absorption	calcium butyrate	poultry, piglets	encapsulated
	gut epithelial integrity and antibacterial	tributyrin	poultry, swine, rabbits, ruminants	esters
	antibacterial	organic acids, polyphenols	swine, poultry, rabbits	
	antibacterial and gut epithelium integrity	caprylic, pelargonic, capric, undecylic, lauric	swine, poultry, calves	
	antibacterial and gut epithelium integrity	lauric		
	gut health promoter	sodium butyrate	swine, poultry, pre-ruminants, rabbits, pets	protected
	antibacterial, epithelium and immunity	MCFA, lactic	swine, poultry	
	antibacterial and immunity promoter	MCFA, high in lauric	swine	
	antibacterial, epithelium and immunity	MCFA, non-fermentable soluble fibre	swine	
	antibacterial	MCFA, lactic, natural polyphenols, two functional fibre types	swine	synergy
	gut microbiota modulator	organic acids protected, synthetic clay, natural vegetal extracts	swine, poultry, rabbits	synergy, protection
	gut microbiota modulator	MCFA protected, butyric, synthetic clay, natural vegetal extracts	swine, poultry	synergy
	antibacterial and gut health promoter	phosphoric, formic, propionic, lactic, calcium lactate, lignosulfonic, essential oils, propylene glycol, calcium butyrate, SiO_2	multispecies	synergy, powder or liquid
	nutrient absorption	calcium butyrate, fumaric	monogastrics, ruminants	microencapsulated
	gut health and nutrient absorption	calcium butyrate, fumaric, essential oils	monogastrics, ruminants	microencapsulated, synergy
	gut health	malic, fumaric, essential oils	poultry	microencapsulated
	gut health	sodium formate, fumaric, essential oils	swine	microencapsulated
	nutrient absorption	propionic, NH_3-propionate	monogastric	liquid
	gut health	MCFA, essential oils		synergy, liquid
	epithelium barrier and gut microbiota modulator	sorbic, vegetal fatty acids, MCFA, butyric protected, salt-edible fatty acids	poultry, swine	

2.2.1 Growth enhancement

When testing any in-feed additive, one of the main outcomes used to be assessed is their ability to improve performance. Improvements in performance are not only one or the first targets for any producer, but also can be considered the "gold-standard" to assess the potential of the new additive to improve welfare and health, particularly in challenging periods like weaning. From a total of 87 reviewed papers testing the supplementation of SCFA or MCFA alone or in combinations in pigs, 62 measured the average daily gain (**ADG**) (Figure 2.4) and 51 the gain-to-feed ratio (**GF**) (Figure 2.5). At first glance, general positive results were observed with increments of ADG and GF. Improved growths range from +1 to +102 g/day of ADG, while the worsened ADG reported, although fewer, range from -1 to -117 g/day. For GF, excluding values measured for animals at suckling, most of the works also displayed better GF ranging from +0.001 to +0.459, but also were reported worsened GF reductions from -0.005 to -0.160.

Figure 2.4 Average daily gain (ADG) differential between treated group and the correspondent control group among 62 published papers. All treatments were based on SCFA or MCFA individual formulas or in combination with other compounds. Legend: △ suckling, ○ weaning, or □ fattening; ● formic and derivatives, ● propionic acid, ● butyric and derivatives, ● caprylic acid, ● capric acid, ● SCFA mixture, ● MCFA mixture, ● SCFA+MCFA mixture, or ● combination with other organic acids (OA) or natural compounds. [+] Indicates animals challenged with *Salmonella* spp. or enterotoxigenic *Escherichia coli* (ETEC) F4. The works used to build this graph are listed in Appendix A.

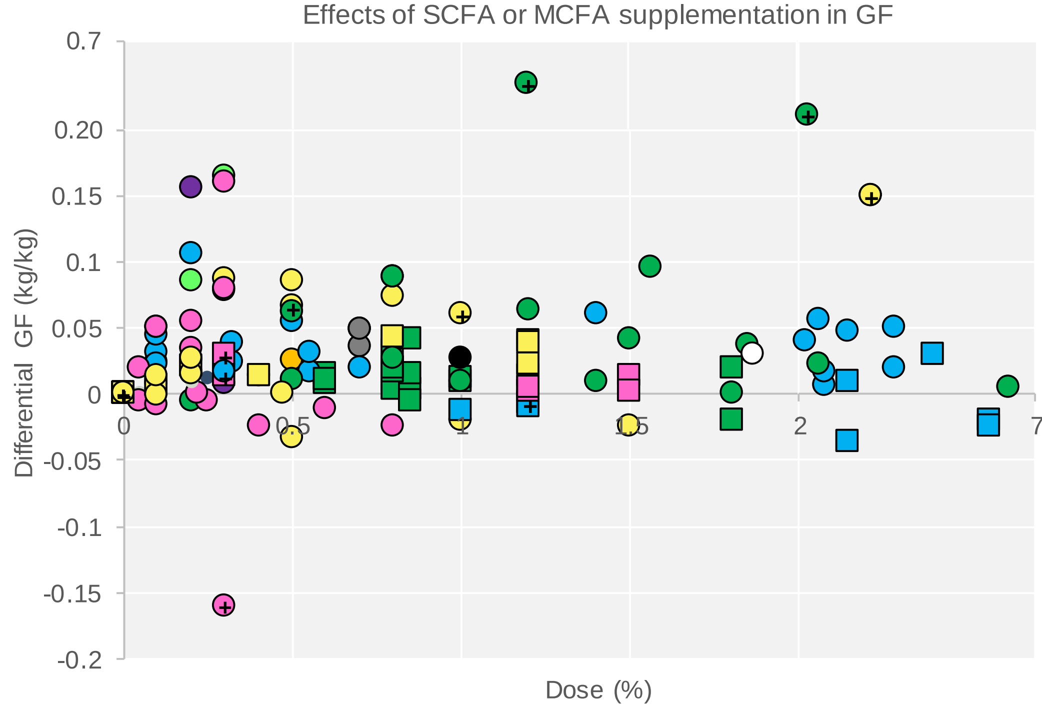


Figure 2.5 Gain-to-feed ratio (GF) differential between treated group and the correspondent control group among 51 published papers. All treatments were based on SCFA or MCFA individual formulas or in combination with other compounds. Legend: ○ weaning, or ☐ fattening; ● formic and derivatives, ● propionic acid, ● butyric and derivatives, ● caprylic acid, ● capric acid, ● SCFA mixture, ● MCFA mixture, ● SCFA+MCFA mixture, or ● combination with other organic acids (OA) or natural compounds. + Indicates animals challenged with *Salmonella* spp. or enterotoxigenic *Escherichia coli* (ETEC) F4. Results from suckling animals were excluded. The works used to build this graph are listed in Appendix A.

Most of the works have been focused on the study of supplementing any of the C1-C12 fatty acids in nursery diets, especially those based on formic acid and derivatives as well as butyric acid or butyrate salt. Formic acid and derivatives have been extensively studied, with general positive results in performance. In nursery piglets, several authors found that formic or derivatives (potassium formate or diformate) by administering doses up to 6.4 % had improved ADG performance (Manzanilla et al., 2004; Kil et al., 2006; Partanen et al., 2007; Poeikhampha & Bunchasak, 2011; Li et al., 2008; Luise et al., 2017) as well as under challenging conditions (Bosi et al., 2007; Htoo & Molares, 2012). Nonetheless, other works did not find these improvements (Canibe et al., 2001; Tsiyolannis et al., 2001; Ettle et al., 2004). Among other production phases, considering the entire fattening phase, mostly ADG and also GF of growing pigs could be improved with formic and derivatives at doses between 0.6-0.8 % (Øverland et al., 2000; Partanen et al., 2002a; Canibe et al., 2005; Eisemann & van Heugten, 2007; Øverland et al., 2007; Partanen et

al., 2007; Øverland et al., 2008) usually without affecting carcass characteristics. However, the work Eisemann & van Heugten (2007) also observed a dose effect. Although GF was improved with formic acid supplementation (0.8 %) from nursery until finishing, it worsened at 1.2 %.

In the same line, butyrate has gained if not all, almost a great part of the interest as an additive to support the early weaning (from 2000' onwards). The work of Gálfi & Bokori (1990) has been one of the first evidences of butyrate benefits. Piglets with in-feed supplementation of sodium butyrate at 0.17 % showed greater body weight (BW) at the end of the nursery phase and fattening as well as final better efficiencies than the control diet (not reported in Figure 2.4 and Figure 2.5 due to variable and old genetics from almost 30 years ago -more than 7 months to reach slaughter weight).

Continuing in the same line, the supplementation of sodium butyrate (0.1-0.4 %) in weaned piglets have shown, in general, positive results in terms of ADG or GF (Piva et al., 2002; Manzanilla et al., 2006; Biagi et al., 2007; Mazzoni et al., 2007; Lu et al., 2008; Fang et al., 2014; Huang et al., 2015). However, some results might be cofounded with the simultaneous administration of low doses of antibiotics (Huang et al., 2015; Boas et al., 2016), and even other works did not evidence such improvements in performance (Mallo et al., 2012; Barba-Vidal et al., 2017a). In addition, several limitations have been observed related to the time being administered and the dose. In this regard, Biagi et al. (2007) did not find a proportional growth with increasing doses (0.1-0.4 %), and Weber & Kerr (2008) found that sodium butyrate had no effect on performance at any time post-weaning (14 or 28 days) and that even at increasing doses (as of 0.2 %) ADG started to be depressed. Øverland et al. (2008) also tested 1.5 % of sodium butyrate to finisher pigs, with no differences on performance.

In the case of the time of administration, some authors found that shorter administrations of butyrate after weaning (up to around 14 days post-weaning), but not further, performance was reinforced (Manzanilla et al., 2006; Feng et al., 2018), while other authors from 21 days up to 6 weeks post-weaning (Biagi et al., 2007; Mallo et al., 2012; Fang et al., 2014) did not find improvements on performances with butyrate (0.1-0.6 %). It could be theorised that the time of supplementing could be limited to earlier days after weaning when piglets may be more compromised. In this regard, Lu et al.

(2012) and Le Gall et al. (2009) observed that supplementing sodium butyrate before and/or after weaning improved the ADG and BW of piglets at post-weaning, but no additive effect was observed by administering along the two phases. Similar results were observed by Mazzoni et al. (2008) that only by supplementing sodium butyrate during the suckling, post-weaning ADG and GF were enhanced enough compared to its supplementation along the two phases. On the counterpart, Hanczaksowska et al. (2016a) did not find significant improvements on the growth of piglets supplemented with butyrate from 7 days of life to 27 days post-weaning. Others did neither find benefits administering exclusively during suckling (Xu et al., 2016). Lastly, works focused on extra-compromised scenarios, under *Salmonella* challenges, observed no improvements by supplementing at 0.3 % sodium butyrate in weaners (Barba-Vidal et al., 2017a) nor in fatteners (Walia et al., 2017).

Also, MCFA have been tested mainly in nursery piglets including several presentations: free or triglyceride forms (MCT), mixtures of different MCFA, and combined or not with lipase. Nonetheless, as a general premise, most of the MCFA tested have been combinations of caprylic and capric acids and/or others rather than individually. Positive results were encountered deduced from the Figures 2.4 and 2.5. Moreover, in general, most of the studies assessing early ages supplementation showed better results, probably supporting the immature functions of the weaning piglets. In this vein, with the supplementation of 0.1-4.8 % of MCFA until around 28 days post-weaning ADG (Dierick et al., 2002a; Marounek et al., 2004; Han et al., 2011; Hong et al., 2012; Zhang et al., 2014; Li et al., 2015; Zhang et al., 2016) and GF (Lai et al., 2014; Li et al., 2015; Yen et al., 2015) were increased, although some authors did not observed such improvements also at high doses (Dierick et al., 2004; Hernández & Pluske, 2008). Other works evaluating from 28-day post-weaning onwards (6 weeks) caprylic and capric acids at 0.2-0.3 % (Zentek et al., 2012; Zentek et al., 2013; Devi & Kim, 2014) or even heptanoic and nonanoic acids at 0.3 % (De Smet et al., 2016) did not find precisely improvements either.

Further studies have been conducted with MCFA opening the window of administration from suckling to post-weaning. Despite the scarce works (Hanczakowska et al., 2010; Hanczakowska et al., 2013; Hanczakowska et al., 2016b), earlier administration from suckling to weaning phases of MCFA (0.3-0.5 %), mainly caprylic and capric acids,

increased the weight of animals at post-weaning phase (21-28 days) to a greater extent than those previously cited only supplementing after weaning. Other authors supplementing high doses of MCT (5-7 %) exclusively during the pre-weaning period did not meet improvements (Trevisi et al., 2017) and even worsened ADG at the moment of weaning (Miller et al., 2016).

2.2.2 Effects on feed intake

Feed intake, especially at weaning, is a concern due to the main threat that represents a reduced or null consumption of solid diet after weaning (Geng et al., 2016a; van der Aar et al., 2017). In this regard, one of the drawbacks attributed to the high levels of supplementation of dietary acids would be their possible negative impact on feed intake. In general, reviews have reported a negative effect on palatability of acids (Mroz et al., 2006; van der Aar et al., 2017). However, when considering a total of 49 reviewed published works, containing a total of 57 experiments, reporting the response of feed intake of SCFA or MCFA supplementation (Figure 2.6), we cannot conclude a negative impact on the intake of acids. Differently from growth responses, in general terms, consumption was not observed to be increased or decreased, but results were displayed quite symmetrically around the 0 value of the y axis.

Variable results were observed in nursery piglets as the main target of the study of these additives. Although the acceptability of piglets for this kind of additives has been a matter of discussion, a lower number of studies reported the feed consumption. Results related to formic acid are inconsistent. Most of the studies did not find differences with formic acid and derivatives in average daily feed intake (**ADFI**) (Ettle et al., 2004; Manzanilla et al., 2004; Kil et al., 2006; Partanen et al., 2007; Li et al. 2008; Htoo & Molares, 2012), while other works showed no preference for formic acid related to the negative organoleptic traits (Ettle et al., 2004; Partanen et al., 2002b; Eisemann & van Heugten, 2007). In older animals, however, formic acid did not affect the feed intake (Øverland et al., 2000; Eisemann & van Heugten, 2007; Øverland et al., 2008). Nonetheless, other works found enhanced ADFI of healthy weaning piglets (Tsiloyannis et al., 2001; Luise et al., (2017) with this acid regardless of the dose (>1 %). In the case of butyrate, some

authors observed a good acceptance and increased ADFI with its supplementation (Lu et al., 2008; Mazzoni et al., 2008; Han et al., 2011), despite most of the results showed no changes (Piva et al., 2002; Manzanilla et al., 2006; Biagi et al., 2007; Mallo et al., 2012; Fang et al., 2014; Huang et al., 2015) and even some showing reduced ADFI with increasing doses (0.05 to 0.4 %) (Weber & Kerr, 2008).

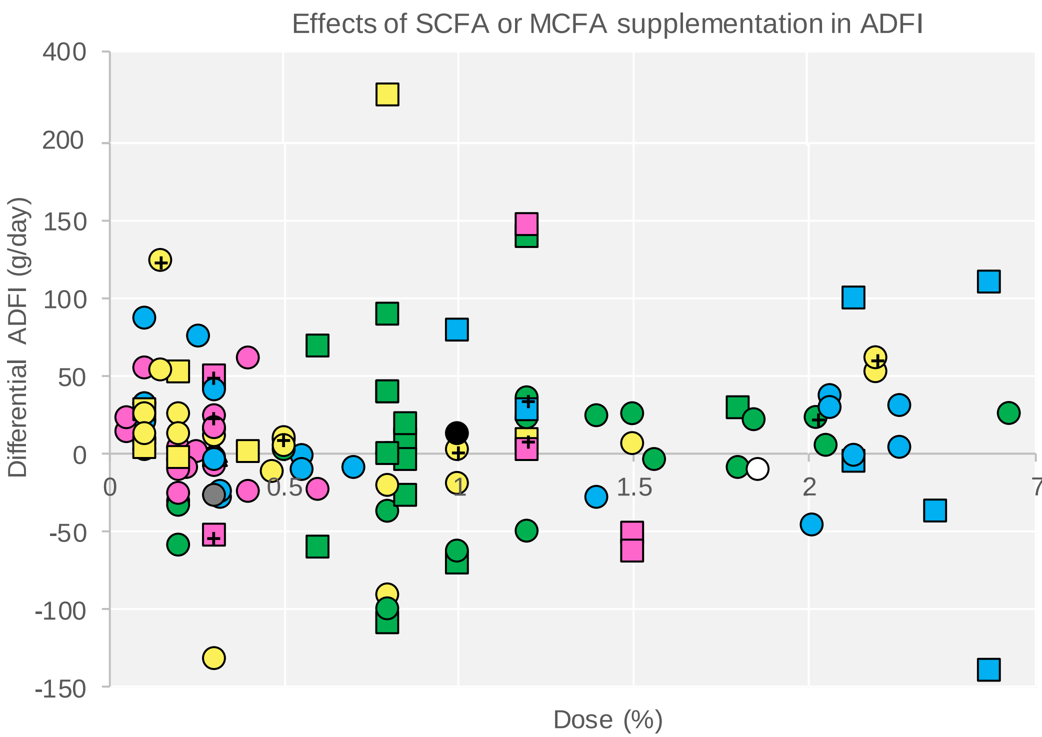


Figure 2.6 Average daily feed intake (ADFI) differential between treated group and the correspondent control group among 49 published papers. All treatments were based on SCFA or MCFA individual formulas or in combination with other compounds. Legend: ○ weaning, or □ fattening; ● formic and derivatives, ● propionic acid, ● butyric and derivatives, ● caprylic acid, ● capric acid, ● SCFA mixture, ● MCFA mixture, ● SCFA+MCFA mixture, or ● combination with other organic acids (OA) or natural compounds. + Indicates animals challenged with *Salmonella* spp. or enterotoxigenic *Escherichia coli* (ETEC) F4. The works used to build this graph are listed in Appendix A.

In the case of MCFA, despite they have associated unpleasant odour and taste (Decuypere & Dierick, 2003), only Li et al. (2015) evidenced the aversion at increasing doses (0.7 to 2.1 %) with caprylic and capric triglycerides given to nursery pigs. However, most of the times, negative effects on ADFI usually did not reach significant levels neither in nursery piglets (0.1-3 %) (Dierick et al., 2002a; Han et al., 2011; Hong et al., 2012; Zentek et al., 2012; Zentek et al., 2013; Devi & Kim, 2014; Zhang et al., 2014; Yen et al., 2015) nor in fattening pigs (Lai et al., 2014; Miller et al., 2016).

Based on the cited works, most of the results reviewed above showed SCFA, especially formic acid and butyrate, as well as MCFA to favour the growth of the piglets at the critical period of weaning with virtually no changes on consumption patterns. Nonetheless, an intense research field involves the relation of fatty acids, especially SCFA and MCFA, in the regulation of the appetite, mainly as a consequence and derivation from human medicine research. This area will be further reviewed in the following section.

2.2.3 Regulation of satiety and impact on metabolism

The dietary fatty acids can prompt the modification of the feed intake and satiety through the interaction with the enteroendocrine system and their hormones. These in turn may affect the metabolism of fatty acids, lipogenesis and lipolysis. Regarding SCFA, these are natural actors in the gut physiology. Contrary to humans (Larraufie et al., 2018), in pigs, SCFA have not evidenced to stimulate enteroendocrine cells to produce the gut hormone peptide YY (PYY), which reduces consumption (Ingerslev et al., 2017). Nonetheless, other studies aiming to decipher the relation between SCFA and the appetite hormones response have been performed.

Interestingly, He et al. (2018) evaluated leptin response of sows supplemented with sodium butyrate and discussed that the lower leptin amounts in the colostrum could be caused by an inhibited production in the mammary gland, what might have permitted the offspring to grow faster. Supplementation of SCFA in growing pigs showed to reduce the feed intake and in turn the BW through the modification of the hormones leptin (increased) and insulin (decreased). Also, SCFA regulated the fat metabolism, depressing lipogenesis by downregulating the fatty acid synthase (FAS) and the peroxisome proliferator-activated receptor (PPAR) in the liver and fat tissue and enhancing fatty acid oxidation by upregulating the carnitine palmitoyl transferase 1 alpha (CPT-1α) in the liver and fat (Jiao et al., 2018). However, the same reasoning has been given for the opposite results with the study of the fat metabolism of piglets supplemented with butyrate, with associated improved energy metabolism and thus, greater BW (Lu et al., 2012). Possibly the age of the animals and the fat metabolism capabilities would have been more

determinant to understand these differences either in hormone regulation and fat metabolism mechanisms.

Considering MCFA, studies in rats and humans have also shown their relation to feed consumption via hormonal and metabolic pathways. Enteral administration of MCT of caprylic and capric acids did not change the appetite hormones leptin or PYY (Ooyama et al., 2009) nor cholecystokinin (Symersky et al., 2002). Uneven-chained MCFA have been shown to induce appetite by the acylation of ghrelin, a gastric hormone released from endocrine cells of mucosa, mainly from stomach, that also regulates fat mass deposition, growth hormone (GH) and insulin release (Nishi et al., 2005; Heppner et al., 2012). Also, the satiety hormone implied might be insulin, affected after MCT supplementation (Ooyama et al., 2009). Miller et al. (2016) evaluated the response of ghrelin and insulin in piglets after adding coconut MCT oil to nursery diets. The authors showed that ghrelin and insulin concentrations increased with increasing doses (from 1 to 6 %), without effects on performance. Nonetheless, the review of Geng et al. (2016a) specifically discussed the supplementation of MCFA effects on pigs. In view of the contradictory outputs, the authors concluded that the equilibrium between the dose of MCFA and the activation of each one of the hormones would have been taking place as well as defined by the physiological status of the animals. At higher doses, hence, MCFA might decrease performance, while supplemented at lower doses might exert the opposite effect.

As described for SCFA, MCFA also have a role in the influence of the systemic energy balance (Geng et al., 2016ab) exercising an inhibitory effect in the liver through the increase of the hepatic oxidation (Jambor de Sousa et al., 2006; Ooyama et al., 2009). In this vein, MCFA have been assessed in piglet models as a rapid source of energy for the survival of new-born babies and for metabolic-inflammatory disorders (Odle et al., 1992; Lee & Chiang, 1994; Wall et al., 1994; Turner et al., 2016). In duodenum-fistulised pigs, MCFA (caprylic and capric triglycerides) showed to be absorbed rapidly, detected in portal vein at 55-65 % and being completely used by the liver (Guillot et al., 1993).

The supplementation to growers of 10 % of coconut oil, rich in MCFA, also inhibited the lipogenesis in the adipose tissue by downregulating the FAS, with no changes in the performance (Iyer et al., 2012). On the other hand, Zhang et al. (2016) found that an early nutritional intervention based on MCT (4 % of caprylin and decanoin) for intrauterine

growth restricted piglets (IUGR) upregulated the hepatic PPAR and thence improved growth, as well as increased adiponectin and ghrelin hormones. However, the replacement of soybean oil in milk formula (21 % fat) with MCT in younger animals (12 days old), with impaired β-oxidation, did not influence hepatic function but even impaired the weight of the piglets (Peffer et al., 2005). Within these results, as was discussed with SCFA, MCFA supplementation in pigs might trigger different performative response in animals depending on the age and therefore their metabolic efficiency, as well as the concentration used of the fatty acid might influence.

In general, there seems to exist a narrow equilibrium whereby the dose of the fatty acid supplemented may regulate the appetite hormones, in close association to fat metabolism. These effects have been studied basically with nutritional levels of MCFA or at doses mimicking naturally-occurring concentrations of SCFA in the hindgut, as a mirror of the human medicine. In this regard, young rather than adult pigs might be benefited because of the advantage of a rapid energetic source when supplemented at proper dosages.

2.2.4 Motility and nutrient utilisation

The study of the gastrointestinal physiology already started in the 70's being human and dog the subjects. On one hand, saturated fatty acids, from acetic (C2), to caproic (C6) acids, including also lauric acid (C12), and long-chain fatty acids (≥C14), were observed to be able to delay the gastric emptying especially with increasing the chain length (Hunt & Knox, 1968; Cooke, 1975), with further impact on motility and hormonal and neural signals from the small intestine. At the turn of the 20th century, works based on pig models aided in elucidating these mechanisms. SCFA have been demonstrated to participate in the ileal brake, i.e. in inhibiting the stomach motility, and thus the stomach emptying through the stimulation of SCFA in the terminal ileum at a concentration-dependent response. Two mechanisms were purposed for the signalling, an indirect humoral response through PYY release, controversial in pigs as was mentioned above, and/or the desensitisation of the vagal afferents from the ileum mucosa through the contact with the SCFA (Cuche & Malbert, 1999; Cuche et al., 2000; Cuche et al., 2001).

On the other hand, acetic, propionic, and butyric acids showed, in earlier works, to stimulate the pancreatic exocrine secretion to different extent (Harada et al., 1986).

The slowed gastric emptying would aid positively in the digestion of the chime and hence, less substrate achieving the lower parts of the gut, especially advantageous for the weaned piglet with underdeveloped digestive function (Michiels et al., 2013). Some authors were capable of demonstrating such impact. Le Gall et al. (2009) demonstrated to improve faecal digestibility and increase the higher dry matter (DM) content in the stomach of weaning piglets, with associated better growth, with the supplementation of sodium butyrate (0.3 % of daily intake of milk DM) in the suckling period. In turn, sodium butyrate (0.3 %) has shown to stimulate the differentiation of gastric mucosa and gastrin expression (Mazzoni et al., 2008) and to improve nitrogen efficiency utilisation with the measure of the faecal urea and amino acids at even lower doses (0.1 %) (Fang et al., 2014). Further works showed some positive results in terms of stomach DM and digestibility with formic (0.2 to 1.86 %) and propionic acids (2.5 %) given to nursery pigs and fistulised adult pigs (Thacker et al., 1992; Manzanilla et al., 2004; Kil et al., 2006). However, some other authors observed non or poorer results in digestibilities or gastric emptying at similar doses with either butyrate in weaners (Manzanilla et al., 2006; Boas et al., 2016) or formic acid in fatteners (Mroz et al., 2002; Canibe et al., 2005).

Regarding MCFA, the MCT are ketogenic in contrast to long-chain fatty acids (Miles et al., 1991), and in dog and human subjects, ketone bodies have been demonstrated to stimulate the protein synthesis when administered (Umpleby et al., 1988; Crowe et al., 1989). MCT, therefore, can provide an energy supply and limit the expenditure of protein as a source of energy (Li et al., 2015), because nitrogen retention might occur when the effective calories in the diet are incorporated. The work of Straarup et al. (2006) found that supplementing triacylglycerols (TG) from coconut oil at 10 % in a pig model of the premature infant improved the fat digestibility during the post-weaning and piglets retained nitrogen, despite the growth was unaffected. Lower doses (0.2-2.1 %) of MCFA (from caproic to capric acids), however, were enough to also improve the energy, fat, nitrogen and protein digestibility as well as to reduce the plasmatic urea related to better performance during early weaning (approximately 2 weeks) (Han et al., 2011; Hanckazowska et al., 2011; Hong et al., 2012; Devi & Kim, 2014; Li et al., 2015).

2.2.5 Stomach barrier

The study of the stomach in the pig has been for a long time related to the poor capability of producing hydrochloric acid in the immature animals at weaning (Cramwell, 1985), what limits the digestion of the dietary protein (Suiryanrayna & Ramana, 2015). If undigested protein surpasses the gastric barrier, it can be fermented in the hindgut and lead to the rise of proteolytic bacteria, including potential pathogens, under a less selective environment (Pieper et al., 2016). In the same vein, the acid-binding capacity (**ABC**) (i.e. the ability to catch protons released from the gastric mucosa) of the ingredients used for weaning diets, like proteins such as whey, or casein, is high and therefore can lower the acidifying ability of the stomach. Some authors propose the formulation of diets based on the ingredient ABC, considering the addition of organic acids (Lawlor et al., 2005; Batonon-Alavo et al., 2016).

Acids have negative ABC values, therefore, their power depends on the capability on releasing protons, ranged as follows for SCFA: formic > acetic > propionic, independently of the protein content or the type of cereal of the diet (Lawlor et al., 2005; Batonon-Alavo et al., 2016). However, in gastric contents, as observed in intestinal contents, pH remains unaffected, or even sometimes increased with either SCFA (formic and diformate, or butyrate) at 0.3-1.8 % (Canibe et al., 2001; Manzanilla et al., 2004; Canibe et al., 2005; Franco et al., 2005; Manzanilla et al., 2006; Biagi et al., 2007; Hanczakowska et al., 2016a) or MCFA or MCT mainly at 0.2-2.5 % (Dierick et al., 2002a; Hanczakowska et al., 2010; Hanckazowska et al., 2011; Hanczakowska et al., 2013; Zentek et al., 2013; Hanczakowska et al., 2016b). In fact, the general discussion regarding the acidification property poses the attention on the weakness of these acids (pK_a of 3-5) (Dibner & Buttin, 2002), with acid constants that might be too narrow to the gastric pH of weanling piglets. Furthermore, they are usually supplemented in the salt form for safer manipulation and better palatability with precisely higher ABC (Lawlor et al., 2005), hence, acidification would be not expected.

Actually, these acids are absorbed in the undissociated form, and in this sense, gastric epithelium has been also studied related to the supplementation of these compounds. Mazzoni et al. (2008) observed a general oxyntic gland development with sodium butyrate supplemented at 0.3 % in weaners, as well as gastric mucosa thickness, and

fundic somatostatin cells (Mazzoni et al., 2008). Also, another work with calcium formate given to weaners (1.2 %), parietal cell numbers and H^+/K^+-ATPase gene expression decreased in the porcine oxyntic mucosa (Bosi et al., 2006), but were unaffected with formic acid in the work of Luise et al. (2017). Actually, stomach deserves further study since it is a place where SCFA and MCFA are mainly absorbed (Dierick et al., 2002a; Manzanilla et al., 2006; Willamil et al. 2011; Zentek et al., 2012). Within this context, although it is generally acknowledged that the largest microbial population of the pig is localised in the large intestine, shifts on gastric microbiota might deserve more insights to study the barrier function of the upper GIT during the post-weaning period. As Canibe et al. (2005) stated, stomach is the major place where microbial and fermentation effects take place due to the diet.

2.2.6 Intestinal barrier

The intestinal epithelium is the responsible of absorbing the luminal nutrients and limiting the entrance of potential pathogens or antigens. In this sense, butyric acid (or butyrate) has been very well documented to directly stimulate the cell proliferation of the gut epithelium (Kien et al., 2007; Le Gall et al., 2009; Lacorn et al., 2010). Also, butyrate has shown to prevent the translocation of commensal bacteria (Lewis et al., 2010) as well as to regulate and enhance the barrier function of the colonocytes by assembling and organising the tight junctions through the activation of the AMP-activated protein kinase (AMPK) (Peng et al., 2009), protein kinase B (PKB or Akt) and receptor GPR109A (Feng et al., 2018).

Works supplementing sodium butyrate to weaned piglets had associated better performances to longer villi and crypts, from jejunum to caecum (Gálfi & Bokori, 1990; Manzanilla et al., 2006; Lu et al., 2008). In the study of Nakatani et al. (2018), they observed that after weaning, the monocarboxylate transporter 1 (***MCT1***) was upregulated in caecum, what would aid in the absorption of the SCFA of piglets, although occludin (***OCLN***), a protein from the tight junctions, was downregulated. Nonetheless, as a general premise, butyrate may reinforce the intestinal barrier by reducing the permeability with the upregulation of tight junctions (*OCLN* and claudins -***CLDN***) in the

small intestine and colon (Fang et al., 2014; Huang et al., 2015; Feng et al., 2018), as well as regulating mucin expression and production via histone deacetylase (HDAC) (Gaudier et al., 2004; Manzanilla et al., 2006).

Regarding other SCFA and MCFA, results on intestinal function were not as evident as with butyric acid. Formic acid and derivatives have shown inconsistent results in weaning piglets (Manzanilla et al., 2004; Bosi et al., 2007; Poeikhampha & Bunchasak, 2011; Hanczakowska et al., 2013). MCFA can also be utilised directly by enterocytes as a fuel and thereby support the integrity of the intestine in young piglets (Guillot et al., 1993). Although not all works have shown benefits with the administration of MCFA as an additive (Hanczakowska et al., 2010; Hanczakowska et al., 2016b), others demonstrated promising results. Combining SCFA and MCFA supplemented at 0.2 % in weaners (Li et al., 2018) or capric acid (0.2 %) individually supplemented from suckling to nursery (Hanckazowska et al., 2011) showed to lengthen small intestine villi. In another experiment (Lee & Kang, 2017), capric treatment alone was able to restore the impaired barrier function of the jejunum in piglets *in vivo* at 0.5 % and demonstrated to upregulate the tight junction genes (*zonula occludens-1 -ZO-1-* and *OCLN*) of intestinal porcine epithelial cells (IPEC-J2), although cytotoxic effects were found at increasing concentrations (1 mM) *in vitro*. Also, Martínez-Vallespín et al. (2016) observed upregulation of *CLDN* with caprylic, capric and lauric acids but at higher doses had negative effects on IPEC-J2 cell integrity. Some works, nonetheless, with higher doses of MCT (2.4-5 %) found positive results in the elongation of small intestine villi *in vivo* (Dierick et al., 2004; Miller et al., 2016; Xu et al., 2018a).

2.2.7 Inflammatory-immune equilibrium

Weaning is associated to an immediate inflammation of the gut characterised by the upregulation of pro-inflammatory cytokines (TNF-α, IL-1, IL-6) in the intestine (Pié et al., 2004). The evaluation of butyrate, among other acids, is also focused in the capability to mitigate the inflammatory response. Butyrate has shown to reduce the inflammatory response triggered by *E. coli* and LPS challenges *in vitro* with intestinal cell lines (Lewis et al, 2010; Melo et al., 2016). *In vivo*, piglets orally supplemented with sodium butyrate also

showed reduction of different pro-inflammatory circulating cytokines TNF-α and IL-6 and their downregulation in the small intestine and colon (IL-6, IL-8, IFN-γ, TNF- α) through the nuclear factor-κ activated B cells (NF-κB) and the HDAC (Lu et al., 2008; Le Gall et al., 2009; Wen et al., 2012; Xu et al., 2016; Feng et al., 2018). However, under challenge conditions, such as under LPS (Weber & Kerr, 2008; Weber et al., 2014), or pathogenic ETEC F4 and *Salmonella* Typhimurium (Bosi et al., 2007; Walsh et al., 2012; Barba-Vidal et al., 2018a) challenges, nor butyrate but neither formate or a blend of acetic, propionic and benzoic acids were able to attenuate the rise of serum cytokines nor to depress their expression (IL-1, IL-6 or TNF-α) (Bosi et a., 2007; Walsh et al., 2012; Barba-Vidal et al., 2018a).

Albeit bacterial infections can lead to an uncontrolled immune response and fatal levels of pro-inflammatory cytokines, cytokine production is necessary for signalling both the adaptive and innate immune system (van der Poll et al., 1999). Thanks to SCFA, a delicate balance on pro-inflammatory cytokine concentration exists whereby the local production of pro-inflammatory cytokines (IL-1 and IL-6) in the small intestine does not necessarily imply the alteration of the systemic response (Milo et al., 2002). In the same regard, SCFA and MCFA have displayed a protective function through the upregulation of the expression of host-defence peptides[4] (HDP) (β-defensin and cathelicidin) in IPEC-J2 cells, especially susceptible to butyrate (Zeng et al., 2013). Also, in the work of Fang et al. (2014), antibody levels were measured, and sodium butyrate increased IgG in serum and IgA+ expression in the jejunum of weanlings.

Regarding MCFA, these also can induce the expression of HDP in IPEC-J2 cells (Zeng et al., 2013). Also, Suzuki et al. (2013) demonstrated MCFA to be ligands of the inflammatory receptor GPR84 present in polymorphonuclear leukocytes (PMN) and macrophages. Nonetheless, some controversial discussion exists regarding their impact on inflammation in *in vivo* trials. Some authors did not find differences neither in protective nor inflammatory response at doses between 0.1-0.55 % in weaned piglets (Han et al., 2011; Hong et al., 2012). Notwithstanding, MCFA can attenuate inflammation and

[4] Host defence peptides (HDP) are produced by immune cells (such as macrophages and monocytes) and intestinal pig cells as an antibiotic mechanism by the host to kill a wide range of microbes, and also potentiate immune response by recruiting more cells and activating others (Zeng et al., 2013).

improve the stress status. Supplementing either caprylic, capric or lauric acid showed to reduce the expression of cytokines in IPEC-J2 (Lee & Kang, 2017). Trials with piglets have shown also to reduce circulating TNF-α and IL-6 with the dietary supplementation of capric acid (Lee & Kang, 2017), or C-reactive protein (CRP) with MCT supplementation (Turner et al., 2016). Other works, moreover, found that supplementation of MCT (0.7-4 %) reduced the oxidative stress of piglets by measuring, for instance, the hepatic glutathione (GSH) redox cycle metabolites, or plasma malondialdehyde (MDA) (Zhang et al., 2014; Li et al., 2015). Supplementation of MCFA combined with SCFA at lower doses (0.3 %) also induced changes in serum peroxide and hydroxyl radicals (Long et al., 2018).

2.2.8 Antimicrobial effects

Among all the effects of SCFA and MCFA cited until here, there is no other more important than their role as antimicrobials in a context of phasing out prophylactic antibiotics. There is a general agreement in the mode of action related to its dissociation constant (K_a or the logarithm pK_a) (Mroz, 2005). At lower pH, the undissociated form can diffuse across the cell membrane of the microbe where the more neutral pH dissociates the acid and releases the H^+ that will disrupt the cytosol and inhibit the growth by inactivating bacterial enzymes. Within more basic pH, such as in piglet stomach or in the distal gut, these acids may act as acidifiers liberating H^+ and acting as a pH barrier again. However, these assumptions may be constrained within a strictly chemical basis, which probably is too simple considering the complexity of the digesta and the interaction of the gut with the host, feed particles, microbiota ecosystem, and so on. Moreover, as reviewed before, supplementation of SCFA or MCFA rarely evidence acidification of the digesta and are naturally absorbed in the foregut.

Longer-chain fatty acids such as MCFA have been widely assessed *in vitro* because of their antimicrobial activity. Precisely, carbon lengths of ten or twelve (capric and lauric acids) are more pH independent and effective than longer- or shorter-chain fatty acids against gram-positive or negative bacteria and pathogenic such as *Salmonella*, ETEC F4, or *Brachyspira hyodysenteriae* (Petschow et al., 1996; Bergsson et al., 2002; Skrivanová et al., 2004; Batovska et al., 2009; De Smet et al., 2016; Martínez-Vallespín et al., 2016;

Vande Maele et al., 2016). Actually, the mechanisms described for longer-chain fatty acids are due to their surfactant properties that may solubilise the microbe membrane and disrupt essential functions for living located in the membrane, e.g. electron transport chain, or by simply lysis of the cell, among others (Desbois & Smith, 2010). A more visual evaluation of the damage in the membrane has been presented with transmission electron microscope (TEM) (Figure 2.7) of *Clostridium difficile* and *Staphylococcus aureus* (Tangwatcharin & Khopaibool, 2012; Shilling et al., 2013) but also the cytosol was observed to be disorganised in *E. coli* and *Clostridium perfringens* (Skrivanová et al., 2006). Nonetheless, these effects are more complex as was pointed out, since other elements in the media may modulate this activity (Bae & Lee, 2017).

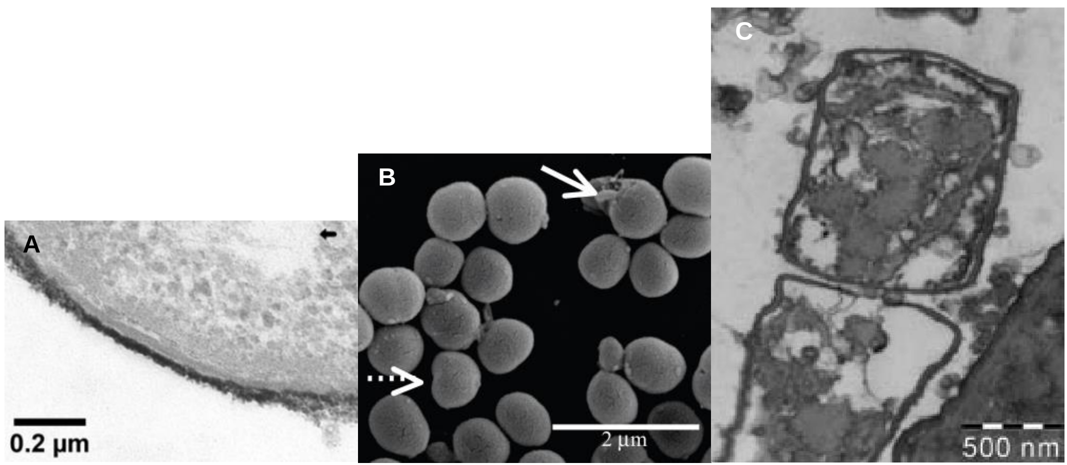


Figure 2.7 Disruption of the cell membrane, vacuoles (arrow), and disorganisation of the cytoplasm of *Clostridium difficile* with lauric acid treatment (A) (Shilling et al., 2013). Leakage (solid arrow) and subsided (dashed arrow) of the cell membrane of *Staphylococcus aureus* with lauric acid treatment (B) (Tangwatcharin & Khopaibool, 2012). Cytosol disorganisation of *Clostridium perfringens* with lauric acid treatment (C) (Skrivanová et al., 2006).

Nevertheless, when evaluating the effects in animal models, most of the antimicrobial effect might be observed in the proximal tract, i.e. stomach and proximal small intestine, since the acidic pH might aid to exert their activity as well as, definitely, is the main site of absorption. In this vein, more recently, the gastric environment and its fermentative activity are receiving attention (Mann et al., 2014; Motta et al., 2017; Almeida et al., 2018; Mailhe et al., 2018). In fact, translated to the stomach ecosystem, it is assumed that higher microbial biodiversity might manifest a better health status since the abundance

and composition of the microbiota and SCFA are suggested to be biomarkers of wellbeing (Ríos-Covián et al., 2016).

In fact, antimicrobial activity might not be as straight as thought and may depend therefore on other abilities described for the acids related to the gastric motility, digestive function or modulatory activity on microbiota ecosystem along the gut. For instance, if formic acid delays the gastric emptying (Manzanilla et al., 2004), it might allow to spend longer contact with the microbiota and thus exert the antimicrobial effect. In this regard, formic acid and derivatives have shown to reduce lactic acid bacteria (**LAB**) counts in the stomach but not to affect *E. coli* (Canibe et al., 2001; Canibe et al., 2005). Moreover, in growing pigs, Canibe et al. (2005) supplementing formic acid showed a reduction in the the microbial diversity in the stomach, including enterobacteria. This fact was related by the authors to the observed increased fermentation in the distal sections of the gut due to the arrival of a higher amount of potentially fermentable digesta to the hindgut. However, on the contrary, some acids may improve the digestive function of the small intestine and thus, limit the available substrate to be fermented in lower parts of the gut. For instance, with sodium butyrate supplementation, weanlings reduced total SCFA in jejunum (Hanczakowska et al., 2016a) and in colon (Manzanilla et al., 2006). Related works of Manzanilla et al. (2006) evaluating the jejunal and colonic microbiota did not show less but higher diversity with butyrate (Castillo et al., 2006; Roca et al., 2014). Furthermore, Franco et al. (2005) did not observe changes on SCFA concentrations of piglets with the administration of formic-based additives but shifts on gastric and small intestinal lactobacilli or coliform populations. The effects of supplemented SCFA on intestinal microorganisms resemble to respond more to complex interactions and not so much to simple direct antimicrobial effects. In this regard, the study of only specific populations may miss others that could play a key role in the effects promoted by these acids.

In the case of MCFA, few works were focused on potential impact on gastric microbiota (Figure 2.8, four studies). No obvious antimicrobial effects were observed, with variable results between studies. While De Smet et al. (2016) almost found no effect in the populations analysed (a minor trend in streptococci to be reduced), Zentek et al. (2012, 2013) showed divergent results with MCFA (caprylic and capric) supplementation to

piglets. In the first work, enterobacteria and clostridia clusters I and IV were incremented (Zentek et al., 2012), whereas in the second work *Bacteroides-Porphyromonas-Prevotella*, and clostridia cluster IV were decreased, also enterobacteria in jejunum (Zentek et al., 2013). Both studies were accompanied with minimum effects on gastric fermentation.

SCFA and MCFA impact on *E. coli*

Differential *E. coli* (log_{10} CFU/g FM)

Dose (%)

Figure 2.8 *Escherichia coli* (log_{10} CFU/g fresh matter [**FM**]) differential between treated group and the correspondent control group among 21 published papers. All treatments were based on SCFA or MCFA individual formulas or in combination with other compounds. Legend: ● including any SCFA, or ● including any MCFA; sto, stomach contents; jj, jejunum contents; ile, ileum contents; col, colon contents. The works used to build this graph are listed in Appendix A.

Regarding the mentioned modulatory effect of these fatty acids, *in vitro* experiments, although limited in mimmicking the entire complexity of the fermentative cavities such as ileum (Partanen & Jalava, 2005; Apajalahti et al., 2009) or caecum (Messens et al., 2010), have shown shifts on specific populations rather than reductions of total bacteria counts or fermentative activity. In fact, as with the works presented until now in this section, most of the works evaluating the GIT microbiota have been focused on specific members, with associated positive/negative connotations. These include: LAB, *E. coli*, *Clostridium*, or *Streptococcus*, among others. It is generally accepted that lactobacilli are important to maintain a good intestinal health because of their ability to control potentially pathogenic groups, such as *E. coli* (Perdigon et al., 2001). Several authors

observed coliform or *Clostridium* populations to decrease in the rectum and faeces of pigs while lactobacilli increased with the supplementation of formic acid and derivatives, regardless of the age of the animals (Øverand et al., 2000; Partanen et al., 2007; Li et al., 2008; Øverland et al., 2008; Hanczakowska et al., 2013). However, these changes were not consistent in other upper segments, e.g. caecum (Li et al., 2008; Poeikhampha & Bunchasak, 2011). With butyrate supplementation, coliform populations were effectively inhibited from jejunum to colon in weaning pigs (Gálfi & Bokori, 1990; Lu et al., 2008) as with MCFA (Hanczakowska et al., 2016b). But again, not always results were so conclusive (Hanckazowska et al., 2011; Lai et al., 2014; Yen et al., 2015; Li et al., 2018). In this sense, again, a systemic antimicrobial effect may be improbable due to the challenging and dynamic the GIT is.

One possible drawback of supplementing acids to control intestinal pathogens would be the possible selection of acid resistant strains that would have more opportunities to surpass the natural barrier of the stomach. Some pathogens such as *E. coli* or *Salmonella* have been described to develop acid resistance mechanisms (Bergsson et al., 2002) to survive in acid pH such as the stomach or in high lactic fermentation environment.

When particularly focusing on pathogen, several published works have tested organic acids using experimental models of disease. Most frequently, they were evaluated under *Salmonella* and pathogenic *E. coli* challenges, always with doses below 1 %. Bosi et al. (2007) and Li et al. (2008) observed *E. coli* to be reduced with potassium diformate in ileum and calcium formate in faeces, respectively, although pathogenic *E. coli* (ETEC) were unaffected. Bhandari et al. (2008) also observed reduction of intestinal populations of *E. coli* with a blend of benzoic, propionic and acetic acids, but did not measure pathogen members. In the case of *Salmonella*, results with fatty acids supplementation are inconsistent. Whereas some studies could demonstrate the reduction of the pathogen in the GIT as well as shedding in weaned piglets with SCFA (0.1-1.2 %) (Boyen et al., 2008b; Taube et al., 2009; Barba-Vidal et al., 2017a), others did not (Martín-Peláez et al., 2010; Michiels et al., 2012). In older animals, butyrate and formic-based blends supplementation reduced *Salmonella* shedding (Creus et al., 2007; Calveyra et al., 2012; Argüello et al., 2013; Walia et al., 2016; Casanova-Higes et al., 2017), although did not prevent the colonisation and the seroconversion prior to slaughter.

Based on the cited works, most of the results reviewed above showed SCFA, especially formic acid and butyrate, and MCFA to favour the piglet's growth at the critical period of weaning, virtually without changes on consumption patterns. SCFA and MCFA can play an important role as microbiota modulators and in the complex interaction with the digestive and immune functions of the animal. Whether their effects are direct or indirect, or limited or not to upper parts it is difficult to decipher. Their local and systemic impact is orchestrated with numerous interactive factors related to diets, health status and hormonal regulatory processes. Furthermore, a fair comparison between studies is unmanageable given the variety of experimental conditions, designs and acids tested. Nevertheless, this review aimed to collect exhaustively all those works reporting performance after supplementing SCFA or MCFA to pigs to bring all doubts to the table.

2.3 Strategies to increase the efficacy of short- and medium-chain fatty acids

As we have seen in the previous chapter, short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) have shown different benefits improving performance and animal health. Despite this, they also have shown some weaknesses that may limit their use in the field and reduce their effectiveness. Between these limitations, we could mention technological aspects associated to their liquid and corrosive nature, their potential toxicity at higher doses, unpleasant organoleptic characteristics of some SCFA and MCFA, and the quick absorption in the foregut that limits their efficacy and activity spectrum in further segments of the gut. Trying to overcome these drawbacks, several strategies and/or technologies have been developed that will be reviewed in the following pages.

2.3.1 Microencapsulation

Encapsulation was originally introduced on biotechnology (60's) to make more efficient the separation of the metabolites within the bioreactor broth from the producer microorganisms or cells. Usually the active ingredients are functional products that are quite susceptible to external conditions (as can be the storage conditions or the gastrointestinal environment) and in this way, they are enveloped, covered and protected by a physical barrier from the outside environment (Nedovic et al., 2011). The term microencapsulation refers to capsule sizes under 1 mm. This form takes advantage of the multiple microparticles expected to be better uniformly distributed; also, multiple doses offer greater safety (Putnam & Garrett, 2005). Although the different hints, encapsulation and microencapsulation are often used interchangeably.

When we talk about animal nutrition, one of the main objectives of encapsulating consists of targeting the delivery towards the low intestinal site by omitting the low pH of stomach and the enzymatic activity in the duodenum (Putnam & Garrett, 2005). Other objectives also include improving safety during handling and/or reducing unpleasant odours.

To be effective against pathogens along the gut, the SCFA and MCFA need to pass the proximal tract of the digestive system without being dissociated before being absorbed to keep their antibacterial activity. Microencapsulating allow organic acids to achieve

distal parts without dissociating completely and therefore also maintaining their efficacy (Gauthier, 2018). In this way, encapsulation guarantees not only SCFA-MCFA bioavailability, but also their functionality in the foregut. Other reasons for encapsulating account for the corrosive acid nature of some of them, and also possible unpleasant odours (Mroz et al., 2005). Especially some have shown negative organoleptic characteristics, e.g. rancid odour for butyric acid or intense goat-like odour for caprylic and capric acids (Mroz et al., 2006; van der Aar et al., 2017). In this regard, some few authors have described in piglets a negative impact on feed intake when increasing the dosage of butyrate or MCT (Weber & Kerr, 2008; Li et al., 2015; Luise et al., 2017). Others have also reported explicit aversion to formic acid and derivatives (Partanen et al., 2002b; Ettle et al., 2004; Eisemann & van Heughten, 2007). Encapsulation should therefore allow to mask this undesired taste and minimise the risk of possible negative impact on feed intake. Nevertheless, it should be said that, despite some negative effects on feed intake reported, most of the times no significant changes were observed on average ADFI (as reviewed in the previous section). The meta-analytic study with OA supplementation in broilers did not confirm such conclusions either (Polycarpo et al., 2017). Beyond palatability, nevertheless, encapsulation can prevent corrosive nature of these acids, dangerous for the handling and irritable for the GIT, as well as can protect them from the susceptibility to lipolysis and rancidity caused by oxidation.

The encapsulation techniques are divided in two groups, chemical or mechanical processing (Parra Huertas, 2010). Chemical processing embraces coacervation, co-crystallisation, molecular inclusion, and interfacial polymerisation, and mechanical techniques include spray-drying, spray-chilling, spray-cooling, extrusion, and fluidised bed (Madene et al., 2006; Parra Huertas, 2010). Encapsulation usually is performed based on spray-drying technology (Madene et al., 2006; Bhandari et al. 2008; Parra Huertas, 2010; Nedovic et al., 2011; Lacerda et al., 2016), since most of the active compounds are liquid, like SCFA, and oily like MCFA (PubChem, 2019). Spray drying (Figure 2.9) is one of the oldest and most extended techniques used since it is flexible, allows a large-scale continuous production, has availability of equipment, yields a stable final product, and is low-cost. Despite these advantages, spray-drying needs to be optimised for each application. With the low optimisation of the process, sometimes the substances are lost

during the spraying (due to their low-boiling points) and we can also have low control over the particle size (Madene et al., 2006; Parra Huertas, 2010; Nedovic et al., 2011).

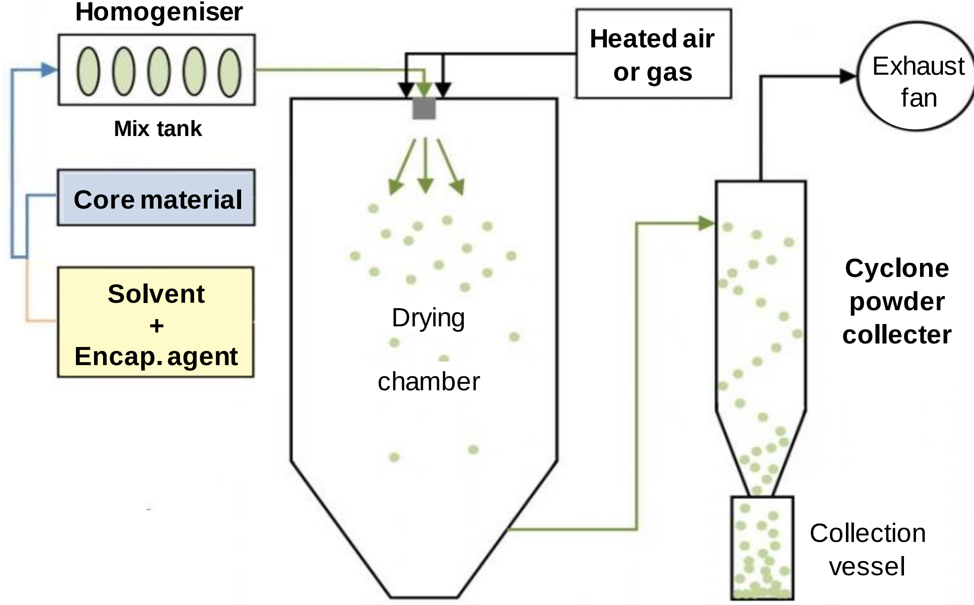


Figure 2.9 Flowchart of the spray-drying process (from Sonarome (2014)). Spray-drying process begins with the liquid solution of the active compound being combined and homogenised with a carrier material and the encapsulating agent that are atomised and sprayed into a heated air stream (drying chamber). Droplets are evaporated and dried instantaneously to form individual particles. Resulting microcapsules are transported to a cyclone separator for the recovery of the free powder (Madene et al., 2006; AVEKA, 2019).

The protection is not intended to last infinitely rather to protect the compound and subsequently release it, conditioned by the temperature, moisture, pH, digestion, etc., to perform the desired function. Precisely, from adaptation from the pharmacological field, the called food-grade delivery systems can vary for our interest herein and from the biological point of view, depending on the site of delivery in the GIT (Benshitrit et al., 2012). These, in fact, can be translated and adapted to each of the challenging situations of mechanical and chemical processes that occur along the GIT related to enzymatic reactions, acid from stomach, and fermentation by colonic microbiota (Gonzalez et al., 2015) that may have deleterious effects on the delivery.

Therefore, to select a proper encapsulation material, the dosage and requirements of the product, like the release characteristics, and the environmental conditions, and the objective to be satisfied have to be considered (Singh et al., 2010). For applications in animal feeds, it is better to use natural polymers that have been approved for use in feeds. Based on the nature of the encapsulating agent, these include protein,

carbohydrate, lipid or mixed systems (Nedovic et al., 2011; Benshitrit et al., 2012; Lacerda et al., 2016). All these natures are applied to spray-drying, although the major types of molecules used are carbohydrate- or protein-based because of the physicochemical properties and the process parameters for spray drying, whereas oils-based (lipid) coating are usually used for spray-cooling/chilling techniques due to the low-melting point (Madene et al., 2006; Nedovic et al., 2011; Lee et al., 2018). Lipid coating agents can be targeted to the small intestine where are degraded by pancreatic lipases and then release solely the active compound (Piva et al., 2007; Khanvilkar et al., 2016). Carbohydrate-based encapsulation takes advantage of the versatile structure and the site-specific digestion of their compounds, for instance polysaccharides, usually plant-derived as starch, inulin, or maltodextrin (Khanvilkar et al., 2016), which in turn can add a bioactivity value as prebiotics (Imran et al., 2012; Lacerda et al., 2016). Protein-based encapsulations are conformed of a variety of different types of substances, with high molecular weights, different molecular chains, and with good film-forming properties (Madene et al., 2006). Nonetheless, the encapsulating material has also to be very precisely designed being, for instance, susceptible to the age of the animal, e.g. younger piglets can be defeated by fat sources that cannot be easily degraded (Pluske et al., 2003), or interestingly, incompatible with the bioactive substance (Kien et al., 2007).

Microencapsulation has been an extensively studied technology of protection (Table 2.3), and the most commonly used among the commercialised organic acids (Table 2.2). The majority of the published works test lipid-encapsulation technology and to lesser extent carbohydrates, while no works were found in literature, up to our knowledge, testing a protein-based encapsulation. Several published works study the capability of encapsulated SCFA and MCFA to impact on distal gut. These include positive effects involved in the control of the intestinal pathogens and the associated diarrhoea, for instance, the reduction of pathogen loads under *Salmonella* challenges, localised in distal parts (Boyen et al., 2008b; De Ridder et al., 2013a; Gebru et al., 2010; Michiels et al., 2012; Casanova-Higes et al., 2017), and the amelioration of post-weaning diarrhoea commonly caused by other intestinal pathogens (Bosi et al., 2007; Fang et al., 2014; Huang et al., 2015; Long et al., 2018), or also the enhancement of the intestinal barrier (Bosi, et al. 2007; Claus et al., 2007; Fang et al., 2014).

Table 2.3 Presentation of all the works testing protected SCFA or MCFA compared classified based on the encapsulating agent.

Delivery systems based on		**Description**	**Main effects**
		Weaning piglets	
Lipid agent	Bosi et al. (2005)	ETEC F4 challenge 0.12 % calcium formate Protection with fat	Growth improvements and reduction of diarrhoea and pathogenic *E. coli* F4. Longer small intestine villi. No differences with unprotected form.
	Bosi et al. (2007)	ETEC F4 challenge 2.2 % calcium formate Microencapsulation with hydrogenated vegetal triglycerides Longer small intestine villi.	Reduction of diarrhoea and faecal *E. coli*. Growth improvements. No differences with unprotected form.
	Bosi et al. (2009)	ETEC F4 challenge 0.6 % sodium butyrate Protection with fat	Trends in growth improvements and reduced deaths after the challenge. Trends in enrichment of faecal *E. coli*. No differences with unprotected form.
	Boyen et al. (2008b)	*Salmonella* challenge 0.2 % calcium butyrate 0.31 % caprylic acid Coating agent fatty acids	Reduction of *Salmonella* excretion and colonisation in lymph nodes only by butyrate. Better than unprotected forms.
	Claus et al. (2007)	0.29 % calcium butyrate Coating with fat	Longer and higher numbers of distal-jejunum villi. No comparable unprotected form.
	De Ridder et al. (2013a)	*Salmonella* challenge 0.3 % calcium butyrate Coating with plant oils	Reduction of *Salmonella* transmission and colonisation in lymph nodes. No comparable unprotected form.
	De Ridder et al. (2013b)	*Salmonella* challenge 0.3 % calcium butyrate Coating with plant oils	No effect on pathogen loads. No comparable unprotected form.
	Fang et al. (2014)	No challenge 0.1 % sodium butyrate Encapsulation with resin	Reduction of diarrhoea and enhance of intestinal immunity. No comparable unprotected form.
	Han et al. (2011)	No challenge 0.1 % eucalyptus extract (mainly caprylic and capric acids) Microencapsulation with palm oil	Growth improvements similar to antibiotics and better faecal digestibility. No comparable unprotected form.
	Mallo et al. (2012)	No challenge 0.25 % sodium butyrate Encapsulation with vegetable fat	No improvements on growth. No comparable unprotected form.

Table 2.3 (continued).

Lipid agent	Upadhaya et al. (2018)	No challenge 0.1 and 0.2 % of fumaric, citric, and malic acids, and MCFA (caprylic and capric acids) Microencapsulation with lipid	Growth improvements and better faecal digestibilities. Reduction of faecal *E. coli*. No comparable unprotected form.
	Weber et al. (2014)	LPS challenge 0.22-to-0.06 % butyric acid Protection with fat	No improvements on growth and no alleviation of LPS challenge. No comparable uncoated form.
	Zentek et al. (2012)	No challenge 0.255 % mixture of caprylic and capric acids Coating with vegetable fat, and sunflower lecithin	No compound detected in distal parts but impact on gastric microbial ecosystem (not antibacterial). No differences with unprotected form.
		Growing pigs	
	Casanova-Higes et al. (2017)	*Salmonella*-infected farm 0.3 % sodium butyrate Coating with vegetable fat	Reduction of *Salmonella* shedding without colonisation in lymph nodes. No comparable unprotected form.
	Gebru et al. (2010)	*Salmonella* challenge 0.2 % mixture of citric, fumaric, malic, and phosphoric acids Microencapsulation with fat	Reduction of *Salmonella* shedding and alleviation of impaired growth. No comparable unprotected form.
	Øverland et al. (2007, 2008)	No challenge 1.2 % calcium butyrate Coating with fatty acids	No effects on growth but only reduction of coliforms in small intestine. No comparable unprotected form.
	Upadhaya et al. (2014)	No challenge 0.1, 0.2 and 0.4 % of fumaric, citric, and malic acids, and MCFA (caprylic and capric acids) Coating with lipid	Improved growth and increased faecal lactobacilli at 0.2 %. No effect on digestibility. No comparable unprotected form.
	Upadhaya et al. (2016)	No challenge 0.1, 0.2 and 0.4 % of fumaric, citric, and malic acids, and MCFA (caprylic and capric acids) Matrix coated with lipid	Improved growth and enrichment of faecal *Lactobacillus*. No comparable unprotected form.
	Willamil et al. (2011)	No challenge 0.25 % lactic and formic acids Microencapsulation with lipids	No detection of compounds in GIT. No effects on performance. In commercial conditions, reduction of caecal enterobacteria and reduction of *Salmonella* shedding at abattoir. No differences with unprotected form.

Table 2.3 (continued).

Lipid agent	Sow/sckukling piglets		
	Devi et al. (2016)	No challenge 0.1 % and 0.2 % of fumaric, citric, and malic acids, and MCFA (caprylic and capric acids) Coating with lipid	No effects on performance of sows nor piglets. In sows, better faecal digestibility, reduction of faecal *E. coli* and reduction of gas emissions (NH_3, H_2S). Improved immunity in sows and piglets. No comparable unprotected form.
	He et al. (2016)	No challenge 0.05 % sodium butyrate Encapsulation with resin	Decreased TNF-α and increased IgA in colostrum. Better growth of piglets. No comparable unprotected form.
	Piva et al. (2007)	No challenge 0.4 % fumaric, malic, citric, and sorbic acids, with vanillin, and thymol Microencapsulated with hydrogenated vegetable lipids (lauric, myristic, palmitic, stearic, and arachidic acids)	Reduction of coliforms in caudal jejunum and caecum and maintenance of LAB and lactic acid concentration. Release along the intestinal tract. Better than unprotected substance.
Carbohydrate agent	Growers/finishers		
	Lacorn et al. (2010)	No challenge 151 g/day sodium butyrate Coating with inulin from chicory (also containing coconut oil)	Longer villi in ileum. Detection of inulin in ileum compared to unprotected form.
	Øverland et al. (2007, 2008)	No challenge 1.5 % calcium butyrate Coating with fatty acids and/or inulin from chicory root	No effects on performance. Only reduction of coliforms in small intestine. Higher butyric acid concentration in colon compared to only fat-protected form.
Others	From weaning to slaughter		
	Partanen et al. (2007)	No challenge 0.8-0.6 % formic acid Diatomaceous carrier	Improved growth at weaning, not the entire cycle. Reduction of faecal β-haemolytic *E. coli*. Better than individual substance.

Table 2.3 (continued).

Non-specified	Weaning piglets		
	Boas et al. (2016)	No challenge 0.1 % sodium butyrate Microencapsulation	No growth nor digestibility improvements. No economically viable. No comparable unprotected form.
	Huang et al. (2015)	No challenge 0.1 % sodium butyrate (with low-dose antibiotic) Encapsulation	Growth improvements and diarrhoea reduction. Improved small intestine permeability and immunity and alleviation of oxidative stress. No comparable unprotected form.
	Long et al. (2018)	No challenge 0.3 % blend of a phenolic compound, slow release lauric acid, butyrate and sorbic acid, MCFA and OA	Better growth accompanied by immunity, and intestinal villi improvements. Reduction of diarrhoea and faecal *E. coli*. No comparable unprotected form.
	Michiels et al. (2012)	*Salmonella* challenge 0.27 % formic, coated sorbic, and benzoic acids 0.18 % caproic and caprylic acid triglycerides and coated oregano oil	Reduction of *Salmonella* with the first blend. No comparable unprotected form.
	Growing pigs		
	Calveyra et al. (2012)	*Salmonella* challenge 0.2 % mixture of fumaric, citric, malic, and phosphoric acids Encapsulation	No effects on pathogen loads. No comparable unprotected form.

However, very few works performed controlled studies comparing the protected and unprotected forms, neither the encapsulating agent alone. When authors have tried to trace the SCFA or MCFA retrieved from the digesta collected from several gut sites (Øverland et al. 2007, Piva et al., 2007; Øverland et al., 2008; Lacorn et al., 2010; Willamil et al., 2011; Mallo et al., 2012; Zentek et al., 2012), no consistent evidence has been found on successful protection as most of the times no significant increase could be found in their luminal concentration. In this regard, Willamil et al. (2011) were not able to detect differences on the amounts of formic nor lactic in the different sites of the GIT with the supplementation of formic and lactic acids within a lipid microencapsulation to grower pigs. However, Piva et al. (2007) found that a hydrogenated vegetable fat-microencapsulating technology was suitable to release inner vanillin and sorbic acid beyond the stomach of piglets compared to the unprotected blend. Moreover, these authors observed a reduction on small intestine and caecal coliform populations. Other authors (Lacorn et al., 2010) also evidenced successful protection testing inulin coating in growing pigs with promising results. They were capable to detect the coating agent (inulin) in the ileum, where villi were actually elongated and *MCT1* upregulated. Authors attributed these effects to the protected substance that was butyrate. Also, by using the carrier diatomaceous earth, an uncommon carrier for organic acids in animal feeding, protected formic acid showed positive results in weaning pigs with improved performance and reduced shedding of β-haemolytic *E. coli* (Partanen et al., 2007).

Together with *in vivo* trials, there are a variety of non-invasive *in vitro* methods suitable for the evaluation of the bioavailability and release of encapsulated compounds, for instance, dissolution tests, or simulating gastric and intestinal fluids (Acosta, 2008). Up to our knowledge, almost no works have resumed these techniques to evaluate the effectivity of protected forms of SCFA or MCFA to be slowly released. Only Omonijo et al. (2018) evaluated lauric acid (with thymol) protected with alginate in a simulated pig salivary (SSF), gastric (SGF), and intestinal (SIF) fluids models showing positive results. Other interesting experiments using these models, although in chicken, were those of Smith et al. (2012) and van den Borne et al. (2015) testing microencapsulated forms of butyrate. In those works, they also combined the use of carbon radiolabelling (^{13}C or ^{14}C) in *in vitro* and also *in vivo* trials, demonstrating the suitability of the protection tested.

2.3.2 Derivatives with other chemical presentations

Some of the aforementioned drawbacks for some SCFA or MCFA, related to their physicochemical characteristics (acidity, volatility or liquid physical form), can be also counteracted by other technological approaches such as changing their chemical form. Between these strategies, we find the salt form presentation and also ester forms.

Solid salts can facilitate the incorporation of SCFA and MCFA to the diets easing the handling as well as reducing the corrosion of the equipments (Mroz et al., 2005). Regarding their palatability, the few studies assessing this trait were mainly focused in formic acid and derivatives, probably because of its especial pungent odour and high corrosiveness (Mroz et al., 2006). However, most of the published works used the acid and the salt terms indistinguishably, perhaps limiting the comparison among studies of the ADFI with the two forms. Ettle et al. (2004) showed piglets to have aversion to formic acid at 1.2 % whereas omitted this problematic with the potassium diformate salt. Eisemann & van Heugten (2007) found that weanlings consumed less proportionally to the dose of formic acid supplied (plus ammonium formate) (from 0.8 to 1.2 %), however, no inference can be done regarding which was the causative agent (acid or salt). Other authors did not achieve to see differences because of the dosage of the acid (1.8 or 6.4 %) (Luise et al., 2017) nor between the acid and the sodium salt (Partanen et al., 2002b). Nevertheless, salt forms can reduce possible aversion responses despite do not necessary gain in preference, as well as salt forms do not necessarily have to show the same activity as the free acid forms either. For instance, by using an *in vitro* ileal fermentation model, Partanen & Jalava (2005) observed that formic acid exhibited a great inhibitory activity on fermentation, while the calcium formate induced the opposite.

Other way to administer SCFA and MCFA is supplementing them as ester forms. Ester linkages can be done with monoglycerides, diglycerides and also with triglycerides. MCFA monoglycerides have been shown *in vitro* to have a greater antibacterial activity compared to free forms. In this regard, Petschow et al. (1996) demonstrated that the monoglyceride of lauric acid, monolaurin, was more bactericidal than the fatty acid against the gram-negative *Helicobacter pylori*. Similar results were observed by Bergsson et al. (2002). Also, Batovska et al. (2009) found that MCFA monoglycerides (caprylate, caprate and laureate) needed lower minimum inhibitory concentrations (MIC) than the

acidic forms against gram-positive pathogens such as *S. aureus* or *Streptococcus pyogenes*, and moreover, combining them at unequal proportions resulted in reduced MIC compared to the substances alone. Tangwatcharin & Khopaibool (2012) also evidenced that monolaurin exhibited the greatest antibacterial activity against *S. aureus* compared to lauric acid or virgin coconut oil. Regarding medium-chain fatty acids as triglycerides (MCT), they have been meant to target distal parts of the gut after pancreatic lipases release the free FA (Zentek et al., 2011). Their combination with lipases also has been tested for the release of FA (Dierick et al., 2002ab), showing interestingly proportional inhibitory effect against microbiota to the fatty acids released *in vivo* (Dierick et al., 2002a). In the same vein, also the triglyceride of butyrate, tributyrin, has been tested *in vitro* again intestinal pathogens such as *Salmonella* not evidencing the same antimicrobial effect as butyric acid (Namkung et al., 2011). However, because tributyrin bypasses the stomach, it has been also purposed as a source of butyrate after its release by lipases in the intestine, which also has been demonstrated to improve intestinal morphology and support the intestinal barrier (Hou et al., 2014; Bedford & Gong, 2018).

2.3.3 Blends and combinations with other additives

The use of acid blends or combinations of SCFA-MCFA with other type of functional additives represents another strategy to improve their activity improving performance and health. Blends take advantage of the synergistic effect observed widely *in vitro*, that in turn allows to reduce the doses and minimise undesirable effects observed when using a unique compound at higher amounts. This approach is called the "multi-hurdle" approach derived from the food technology field (Rostami et al., 2016).

In this vein, the studies *in vitro*, in line with food technology, have focused its objective on evaluating the antibacterial effect of combining these acids against pathogenic species. A proposed parameter to evaluate the synergistic effect has been the fractional inhibitory concentration (FIC) index (ΣFIC) that is calculated as:

$\Sigma FIC = FIC\ A + FIC\ B$,

where FIC A is the MIC of the combination of products A+B divided by the MIC of the compound A alone, and FIC B is the MIC of the combination of products A+B divided by

the MIC of the compound B alone. In this way, synergy is observed when 0.5<EFIC<2 (Ohran et al., 2005).

Combinations between different types of acids, SCFA and MCFA or with OA, are already widely commercialised (Table 2.2) mainly fitting on their capacity as acidifiers. As already mentioned, the concept of food and feed preservatives is imported to animal nutrition, since the pathogens occurring in the aliments are the ones causing animal diseases. In this line, Tangwatcharin & Khopaibool (2012) demonstrated to exert synergistic effect the combination of lauric and lactic acid in a contaminated pork meat model against *S. aureus*. However, other *in vitro* experiments, quantifying the ΣFIC, did not find the same positive results. The combination of lauric acid with butyric acid did not show synergistic effects against the gram-negative spirochete *B. hyodisenteriae* despite alone they were bactericidal (Vande Maele et al., 2016). Neither the combination of acetic with other OA (lactic or citric acids) reduced synergistically *Salmonella* Typhimurium (Nazer et al., 2005). Scaling up, in an *in vitro* ileal fermentation model of pig, combining sorbate and formic showed a greater decline on gas production and VFA concentration than the individual acids (Partanen & Jalava, 2005). Moreover, these changes were associated to the inhibition of certain microbial populations. Also, in an *ex vivo* experiment with jejunal tissue from piglets, the addition of MCFA (caprylic and capric) to OA (fumaric plus lactic) showed to elongate the villi (Ferrara et al., 2017).

In vivo, focused on pathogen control, the combinations studied were also variable. Li et al. (2008) found that a combination of butyric, fumaric and benzoic acids (at 0.5 %) alleviated the growth depression of the *E. coli* F4 challenge in weaned piglets and promoted lactobacilli in the small intestine resembling the colistin control. Considering trials with *Salmonella* spp. challenges, results did not always evidence improvements. In weaner piglets, a combination of formic and propionic acids at 0.9 % did reinforce the stomach barrier and reduce *Salmonella* shedding (Taube et al., 2009). However, the combination of formic with lactic acid at 0.8 % did not reduce the carriage in weaners (Martín-Peláez et al., 2010) but did in fatteners (0.8 %) (Creus et al., 2007; Willamil et al., 2011). Another work with *Salmonella*-challenged growing pigs, Calveyra et al. (2012) did not observe to prevent *Salmonella* transmission by supplementing an encapsulated mixture of fumaric, citric, malic and phosphoric.

Additionally, few studies compared the combinations with the individual compounds (Table 2.4). Once again, the works facing the individual compounds and the blends examined a wide variety of combinations and designs with no conclusive results. This makes even more difficult to compare between studies and conclude which might be the most suitable combination to implement. Most of the works evaluated blends based on formic acid formulas. Partanen et al. (2007) tested in pigs two blends, formic and propionic acids either with adding sorbate or benzoate, with diverging effects during weaning but better growth from weaning to slaughter. During fattening, formic with sorbate showed better results in the performance of finisher pigs, while formic and lactic acids did not counteract the growth impairment of *Salmonella*-defeated animals (Creus et al., 2007). Butyrate was also evaluated in complex mixtures (with lactic, formic and citric acids) in weaning piglets (Boas et al., 2016) or with humic acid under an LPS challenge (Weber et al., 2014). No improvements were seen in performance nor inflammatory response.

As mentioned before, most of the works testing MCFA consisted of combinations rather than the individual acids. The works testing a combination of capric and caprylic acids (0.2 %) from suckling up to weaning, and of caprylic, capric and lauric acids (1 %), exclusively during weaning, showed to impair the performance compared to the individual acids (Marounek et al., 2004; Hanczakowska et al., 2011). Nonetheless, the study of Hanczakowska et al. (2013) supplementing to piglets from suckling to weaning showed better digestibilities and longer villi in the small intestine when propionic and formic acids were combined with MCFA (capric and caprylic acids). In the work of Devi et al. (2016), a different strategy was purposed with positive results. A blend of MCFA (caprylic and capric acids) and OA (fumaric, citric and malic) was tested in a cycle embracing 95 day-gestating-to-lactating sows to evaluate their response and the response of the offspring after the suckling period. In that work, mothers had better faecal digestibilities and declines on faecal *E. coli* and gas emissions (NH_3, H_2S) whereas the immunity was improved in both mothers and piglets, possibly by vertical transfer.

Table 2.4 Presentation of all the works testing of SCFA-MCFA-based blends with other organic acids and/or natural extracts, or lipase compared to the individual compound.

Study	Description	Main effects compared to individual compound
Creus et al. (2007)	Fattening pigs under *Salmonella* challenge 0.8 % formic acid 0.8 % formic and lactic acids (1:1)	No effect on performance. Reduction of seroprevalence and *Salmonella* shedding prior to slaughter, not comparable to formic alone.
Partanen et al. (2002a)	Fattening pigs 0.8 % formic acid 1.2 % formic acid and sorbate	Better growth in finishing. No difference on diarrhoea. Results of both additives similar to avilamycin control.
Weber et al. (2014)	Weaning piglets under LPS challenge 0.06 % butyric acid (fat-protected) 0.25 % humic acid	Decreased cortisol. No effects on growth of healthy animals. No alleviation of systemic inflammation nor oxidative stress under challenge.
Hanczakowska et al. (2011)	Suckling/weaning piglets 0.2 % caprylic acid 0.2 % capric acid	Caprylic improvements pre- and post-weaning on growth, *C. perfringens* reduction and increased ileal villi. No improvements with the combination.
Franco et al. (2005)	Weaning piglets 0.96 % formic acid 1.6 % formic and fumaric acids (1:1) 1.38 % formic and lactic acids (1:1) 1.24 % formic and lactic acids (2:1)	No differences on digestibilities. Impact on gastric fermentation. Only reduction of coliforms in small intestine with the formic and lactic acids.
Marounek et al. (2004)	Weaning piglets 0.5 % caprylic acid 1 % caprylic, capric, and lauric methyl esters	Mixture of methyl esters exhibited poorer growth rates.
Partanen et al. (2007)	Weaning-to-slaughter pigs 0.8-0.6 % formic acid 0.8-0.6 % formic, propionic acids, and potassium sorbate 0.8-0.6 % formic, propionic acids, and sodium benzoate	At weaning, adding sorbate improved growth while benzoate worsened growth and diarrhoea. At finishing, blends improved ADG. From weaning to slaughter, both blends improved ADG. Poorer results compared to avilamycin control.
Boas et al. (2016)	Weaning piglets Lactic, formic, and citric acids Sodium butyrate (microencapsulated) (All diets contained colistin)	No effects on performance nor digestibility.

Table 2.4 (continued).

Xu et al. (2018b)	Weaning piglets 0.15 % benzoic, fumaric acids, and calcium formate (coated) 0.03 % thymol and carvacrol	Better ADG and lower oxidative stress compared to colistin control. No differences on faecal coliforms nor lactobacilli. No general improvement compared to individuals.
Dierick et al. (2002a)	Weaning piglets 2.5 % triglycerides (caprylic, capric and lauric acids) 0.1 % lipase	No effects on growth. Lipase addition released more fatty acids in stomach and intestine correlated to microbiota reduction (total bacteria and *E. coli*).
Hanczakowska et al. (2013)	Suckling/weaners 0.5 % formic and propionic acids 0.7 % propionic and caprylic acids 0.7 % propionic and capric acids 0.7 % propionic, caprylic, and capric acids	Combination with capric acid showed better performance pre- and post-weaning, better protein and fibre faecal digestibilities, and longer villi in ileum.

Works are sorted by the complexity of the blends, first are presented the simplest combinations. Main effects compared to individual substance if not specified. If no blend specified, consider the combination of both presented compounds.

Another strategy to improve antimicrobial activity is to combine SCFA-MCFA with essential oils or natural extracts. In this direction, it is interesting to see the wide offer of microbiota modulator blends commercialised (Table 2.2). In this regard, combining SCOA, sorbic and propionic, with botanicals (eugenol and thymol), a synergistic effect was observed against *Campylobacter in vitro* (Grilli et al., 2013). On the contrary, Vande Maele et al. (2016) did not find synergistic effects by combining butyric and botanicals against *B. hyodisenteriae* despite alone showed bactericidal effect.

Considering trials performed *in vivo* with pigs, some works demonstrated such antimicrobial effects. Manzanilla et al. (2004), adding plants extracts to formic acid (0.5 %), observed reductions in VFA in colon, possibly related to synergistic antibacterial effect. Different results were found, however, in the experiment of Michiels et al. (2012) that tested in *Salmonella*-challenged piglets a battery of several blends (organic acids with natural extracts, SCFA, essential oils, and MCFA) at around 0.18-0.3 %, some alleviating the challenge but others worsening in terms of performance and diarrhoea. Another work with *Salmonella*-challenged grower pigs (Calveyra et al., 2012), a blend of SCFA and plant fatty acids (0.2 %) was not able to prevent *Salmonella* transmission.

Beyond the antimicrobial activity, combining SCFA-MCFA with natural flavours, essential oils, or herbal extracts can stimulate and make animals to be interested in the feed also hiding aversive compounds of the SCFA and MCFA (Clouard & Val-Laillet, 2014). Moreover, there have been described to have effects on the gut and systemic health, e.g. stimulate the immune system, suppress harmful microorganisms, and promote beneficial microbes, and serve as antioxidants (Yang et al., 2015). Actually, some authors (Li et al., 2018; Long et al., 2018) demonstrated more comprehensive benefits with complex blends consisting of classical organic acids (sorbic acid), SCFA, MCFA and phenols (0.3 %) in terms of growth, intestinal barrier, immunity and oxidative stress improvements, and diarrhoea mitigation as well as gut microbiota modulation.

Furthermore, these are not the only compounds being considered. Organic acids have been tested also with other products such as probiotics, or enzymes, with variable results not always being successful. Bhandari et al. (2008) did show slight improvements on growth of weaning piglets challenged with ETEC F4 with a blend of benzoic, propionic and acetic acids with *Bacillus subtilis* and saccharin. Bosi et al. (2009) also evaluated a blend

of butyrate with yeast beta-glucan and mannan-oligosaccharides (MOS) under a similar challenge with improvements on growth but resembling those of butyrate alone. Thacker et al. (1992) did not find improvements after the supplementation in barley-based diets of propionic acid with beta-glucanase despite individual treatments had positive effects in the performance of grower pigs. On the contrary, Lückstädt & Mellor (2011) reported the synergistic effect on performance of combining potassium diformate and phytase, this enzyme probably reducing the ABC of the diet (catching up the phosphate and calcium from diet), and thus favouring the action of the acid.

Summarising, in the literature there can be found different strategies aimed to improve SCFA-MCFA efficacy. Between them, we can remark the microencapsulation and the use of chemical formulas, such as salts or ester forms, to overcome some of the limitations that these acids can have like their quick absorption in the foregut or their corrosiveness or organoleptic traits. Moreover, blends of several SCFA and/or MCFA with organic acids and/or functional compounds, such as phytogenics, have been proved as a way to improve their antimicrobial properties if rational designed. Nonetheless, from the scientific point of view, more insights are claimed to elucidate which is (are) the most suitable strategy(ies) to improve their activity as no conclusive results can be obtained from the scarce number of fully controlled studies.

2.4 Microbiota 16S rRNA gene as a tool of assessment of in-feed additives activity

Gut microbiota exerts a huge impact on physiology and health of the host sustaining a beneficious relation (Figure 2.10) (Gibson & Roberfroid, 1995; Macfarlane & MacFarlane, 1997; Foster et al., 2017). The host supplies substrates for the bacteria such as undigested food, or mucus, as well as compartmentalises the GIT providing different and selective niches for the microbes, e.g. physicochemical traits of the chime, immune pressure to target and discriminate pathogenic from commensal bacteria, and a physical barrier constituted of mucus and tight junctions. The commensal gut microbiota, in turn, exerts a plethora of protective, structural and metabolic effects to the host. The gut microbiota is capable to digest and ferment what the host is unable to degrade and transform it into waste products for the bacteria necessary for the GIT functioning, such as SCFA or lactic acid. The host draws on SCFA as an energy source, especially butyrate for the sustenance of the epithelium, and lactic acid maintains an acid pH keeping out pathogens. Microbe-to-microbe interactions also can limit the presence of pathogens, known as colonisation resistance (CR), by favouring/limiting growth of counterparts by complex cross-feeding networks and competing for nutritional niches, by sending stressing stimulus to the immune system, or by producing antimicrobial substances such as bacteriocins.

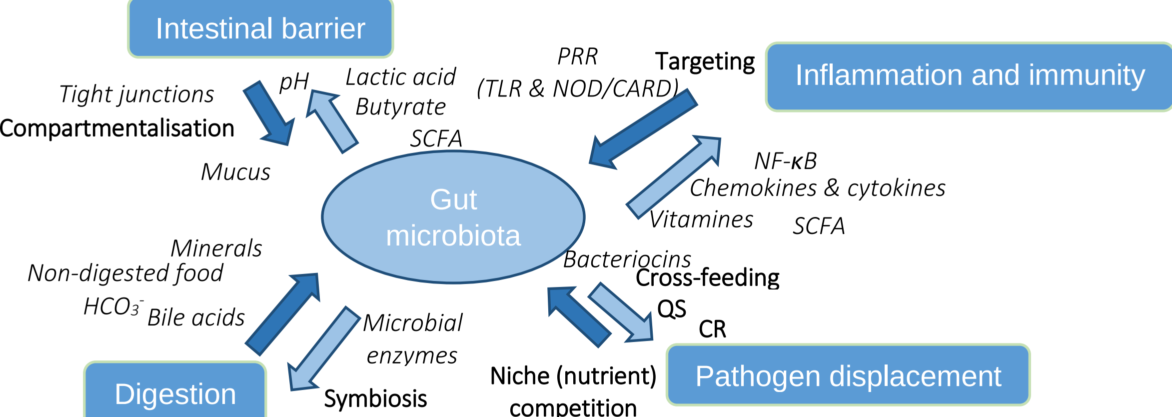


Figure 2.10 Main microbe-to-host (lighter arrows) and host-to-microbe (darker arrows) interactions among gut microbiota and pig (host) depicted in literature (adapted from O'Hara & Shanahan, 2006; den Besten, 2013; Foster et al., 2017; Lustri et al., 2017). Mechanisms in bold, *metabolites* or *signals* in italic. PRR: pattern recognition receptor systems. TLR: Toll-like receptor. NOD/CARD: nucleotide-binding oligomerisation domain/caspase recruitment domain isoforms. NF-κB: nuclear factor-κ of activated B cells. QS: quorum sensing. CR: colonisation resistance.

In view of the crucial role the microbiota plays around the gastrointestinal physiology and homeostasis, the technology to quantify the gut composition is in continuous development and improvement. With the studies of Carle Woese, bacterial 16S ribosomal RNA (rRNA) genes were determined to be used to infer taxonomic assignation for bacteria and archaea, since they were present in all prokaryotes and were composed by multiple conserved sequences flanked by unique less-conserved hypervariable regions capable to discriminate species (Figure 2.11) (Cohan & Koeppel, 2008; Kim & Isaacson, 2015). The first technological approach to study the microbiota ecosystem consisted of cloning the full-length of the 16S rRNA gene after PCR with universal primers and sequencing by the Sanger method, very accurate but time-consuming and costly and with lower coverage of 16S, which only allowed the analysis of dominant organisms (Suau et al., 1999; Blaut et al., 2002; Leser et al., 2002; Weinstock, 2012). In the last years, the development of new massive-sequencing technologies has made possible new approaches for studying intestinal microbiota. This technology is being expanded continuously, currently with next generation sequencing (NGS) platforms. At the moment, the most popular method used to generate the community profile is the 16S rRNA amplicon high-throughput sequencing (HTS) because it is a cost-effective approach and has available pipelines (Sanschagrin & Yergeau, 2014). It consists of the partial sequencing of the gene of the 16S rRNA based on the selection of a single (or combined) hypervariable region of the gene (Figure 2.11) with the final goal of producing a pool of 16S rRNA amplicons proportionally to those of the original sample (Burbarch et al., 2016; Yang et al., 2016; Rintala et al., 2017).

In line with deep human studies and projects to characterise the human gut microbiota (Blaut et al., 2002; HMP, 2019), the characterisation of the gut microbiota of swine has also increased the interest of the research community that now accounts with the support of new technologies at a reasonable cost. In the last years, a deeper knowledge of the pig microbiota has been generated. Now it is known how gastrointestinal (GI) microbiota is a dynamic ecosystem that can respond specifically to aging and to challenges such are weaning, stress, infections, diet and environment and also plays a relevant role in the physiology and immune status of the host. In this way, microbiota has

turned nowadays into an attractive target for dietary interventions to improve the health and the efficiency of the animals.

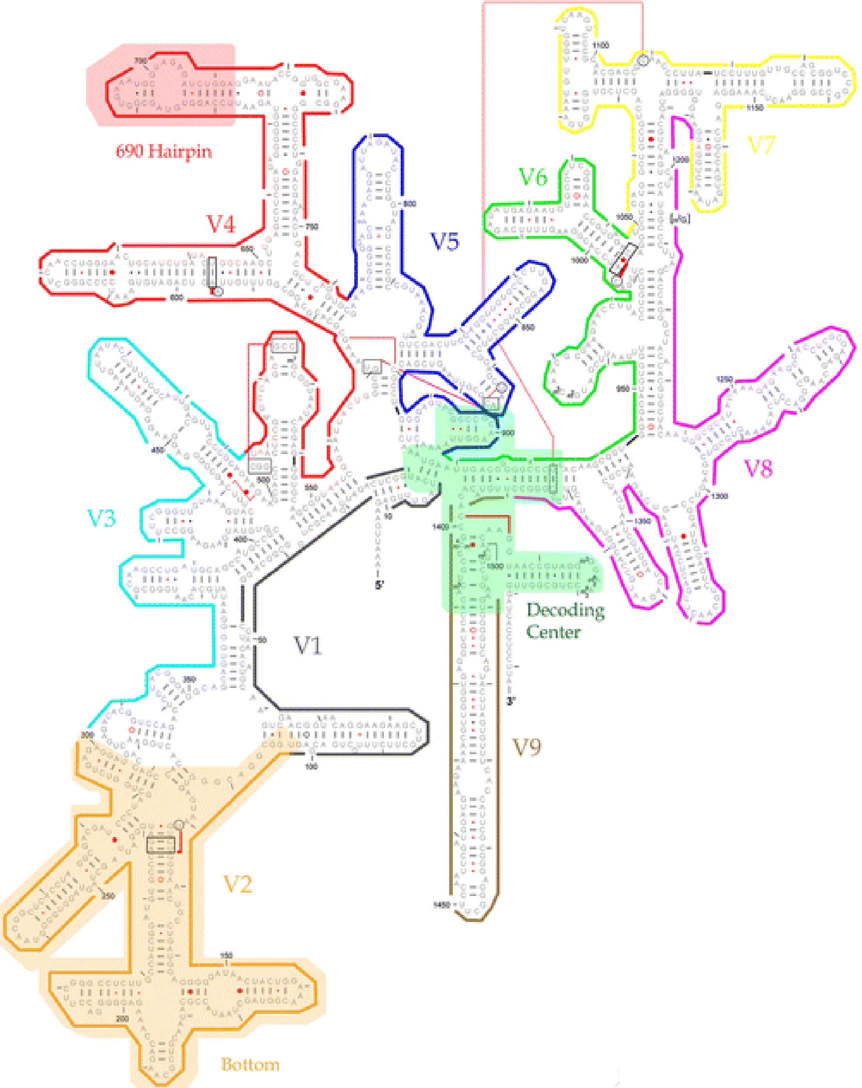


Figure 2.11 16S rRNA 2D structure with each one of the nine hypervariable regions differently coloured (Yang et al., 2016). V4-V6 were determined to represent the optimal sub-regions for the study of the bacterial phylogeny. 690 hairpin is a highly conserved loop crucial for ribosomal functioning (Yang et al., 2016). Decoding centre is another conserved region involved in the decoding genetic codes in mRNA (Zhang et al., 2003).

2.4.1 Changes on pig gut microbiota during first stages of life

From the ecological point of view, an ecological succession is a process whereby specie's structures change in a defined ecological community along the time. It starts with few pioneering species evolving into increasing complex communities until it reaches the *climax community*, which is stable and is self-perpetual (PSU, 2009). In the pig GI

ecosystem, age, as time, is the first natural factor driving bacterial populations into a microbial succession, initiated by a scarcely/un-occupied habitat (Poulsen et al., 2018) that also suffers further restructuration after disturbances such is weaning (Gresse et al., 2017).

There is general agreement in literature relative to which are the main sequential changes in microbial groups from birth to weaning and immediate post-weaning (Figure 2.12). In general terms, facultative anaerobic bacteria are the microbiota starters of the gut being the pioneers Proteobacteria and *Escherichia coli* (Proteobacteria), and Lactobacillaceae, as well as *Clostridium* and *Ruminococcus* (Firmicutes), and *Bacteroides* (Bacteroidetes), amongst others. Also, Fusobacteria and Actinobacteria characterise early ages (Slifierz et al., 2013; Bian et al., 2016; Han et al., 2018). During suckling, microbial communities become more diversified and heterogeneous between animals and the former populations decline in favour of other strict anaerobes, mainly from Bacteroidetes phylum, which involve *Prevotella,* but also other groups such as *Veillonella* (Frese et al., 2015; Mach et al., 2015). In the young pig, two distinct microbial ecosystems have been reported recently, known as enterotypes (Mach et al., 2015). These enterotypes resemble somehow two of the enterotypes previously described for humans (Arumugam et al., 2011) (Figure 2.12). One of the enterotypes described by Mach et al. (2015) was distinctive of suckling piglets, represented by Ruminococcaceae and accompanied by other genera such *Oscillibacter* or *Clostridium* cluster XIV. These groups are adapted to milk compounds like oligosaccharides and natural glycans from the host and have been related to lactose catabolism (Frese et al., 2015) as well as to other compounds such as milk fat and protein (Bian et al., 2016). This type of microbiota has also been described in human as milk-oriented microbiome (MOM) (Zivkovic et al., 2013) that supports the digestion of those glycans that the piglet is unable to degrade (Frese et al., 2015). We could consider therefore, that this enterotype would be associated with better performances during suckling (Mach et al., 2015).

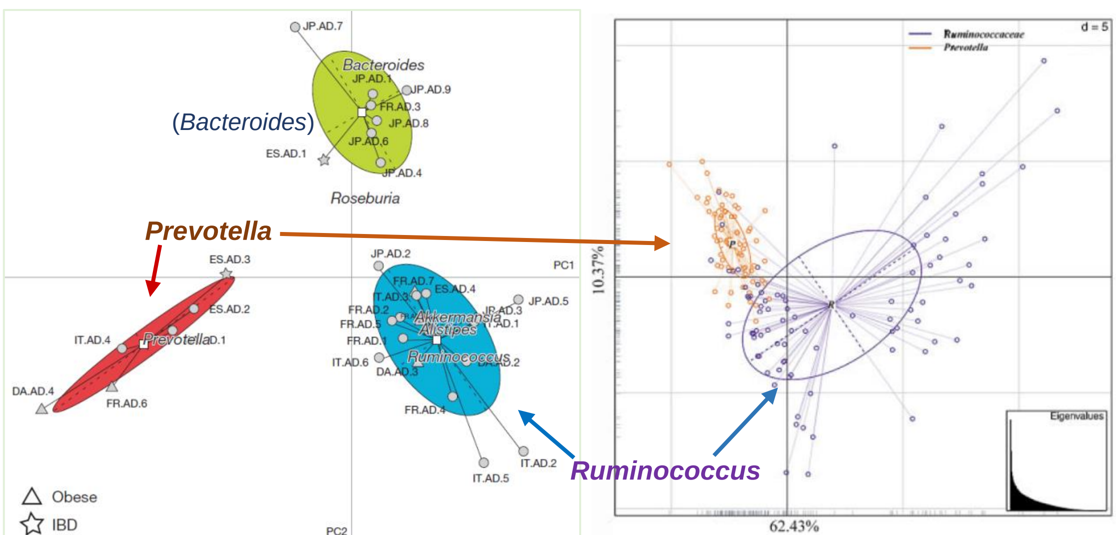


Figure 2.12 Enterotype-like clusters in adult humans from different nationalities consisting of *Prevotella*, *Ruminococcus*, and *Bacteroides* (Arumugam et al., 2011) [left], and in Large White piglets across five age strata (14, 26, 60, and 70 days old) consisting of *Prevotella* and Ruminococcaceae (Mach et al., 2015) [right].

After weaning, microbiota structure becomes more homogenous between animals (Chen et al., 2017) and an intermediate microbiome composition has been observed by several authors, approximately up to 6 weeks post-weaning (Slifierz et al., 2013; Han et al., 2018). This transient microbiota is characterised by the increase of Bacteroidetes and Prevotellaceae as the major populations, accompanied by a higher prevalence of lactobacilli and *Megasphaera* (Kim et al., 2011; Slifierz et al., 2013). Precisely, the second enterotype described by Mach et al. (2015) was related to better growth performances of piglets during weaning and to the predominance of *Prevotella*, also accompanied by *Acetivibrio*, *Oribacterium*, *Paraprevotella*, *Roseburia* and *Succinivibirio* (Kim & Isaacson, 2015; Han et al., 2018). Adaptation to weaning consolidates the colonisation by obligate anaerobes like *Clostridium* or *Megasphaera* that produce butyrate that can support the gut barrier defence as a nutrient for the intestinal epithelium (Slifierz et al., 2013). The work of Ramayo-Caldas et al. (2016) characterised indeed the functionality of the two enterotypes, already presented, by their role on the weaning transition. They showed that the first enterotype-like group displayed functions involved in the metabolism of butyrate, nitrogen and amino acids (for example, alanine, aspartate or glutamate). Being the major driver of this enterotype, Ruminococcaceae, and other co-occurrent bacteria such as *Clostridium*, *Blautia* and *Dorea*, also butyrate producers. Regarding the second enterotype, it is characterised by the predominance of *Prevotella* related to the

degradation of fibrous compounds, suggesting that piglets with this enterotype are capable to meet better growth performances at weaning because their microbiota is better adapted to the new solid diet. In turn, consuming the same diet prompts the more homogenous microbial ecosystems between animals (Ramayo-Caldas et al., 2016). Genuinely, age is intimately linked to the composition of the diet, as the older the animal the better the capacity to digest high-fibre diets (Niu et al., 2015).

In young animals, the intestinal microbiota can be also determined by innate factors, particularly maternal effects, harbouring starting communities with stronger resemblance to their mothers that disappear towards weaning and further on (Frese et al, 2015; Mach et al., 2015; Bian et al., 2016). Mother effect can be indeed cofounding with genetics, or milk composition effects. Nonetheless, comparable microbiota profiles between sows and older pigs have been shown in other works (Figure 2.13), possibly because the objective focused on the adult microbiome rather than the maternal effect. In this line, adult pigs finally approach an "adult profile" characterised by a high Firmicutes:Bacteroidetes (**F/B**) ratio, greater diversity and richness, and a more homogenous and stable microbiota (Kim et al., 2015; Han et al., 2018). Curiously, compared to other animals such as chicken, mouse, or fish, pig gut microbiome has a superior richness but greater homogeneity among animals (Lamendella et al., 2011; Xiao et al., 2016). In addition, there are features of the swine gut microbiota that are characteristic compared to other species. For instance, spirochetes, like *Treponema* (Kim et al., 2011; Lamendella et al., 2011; Bian et al., 2016), or Euryarchaeota (Han et al., 2018) are commonly found in the gut of the adult pig. Hence, considering all these changes in terms of microbiota structure and populations, the profiling of new microbial ecosystems may have a reference, if not exact, guiding of which might be the bacteria normally found in the digestive tract of the pig.

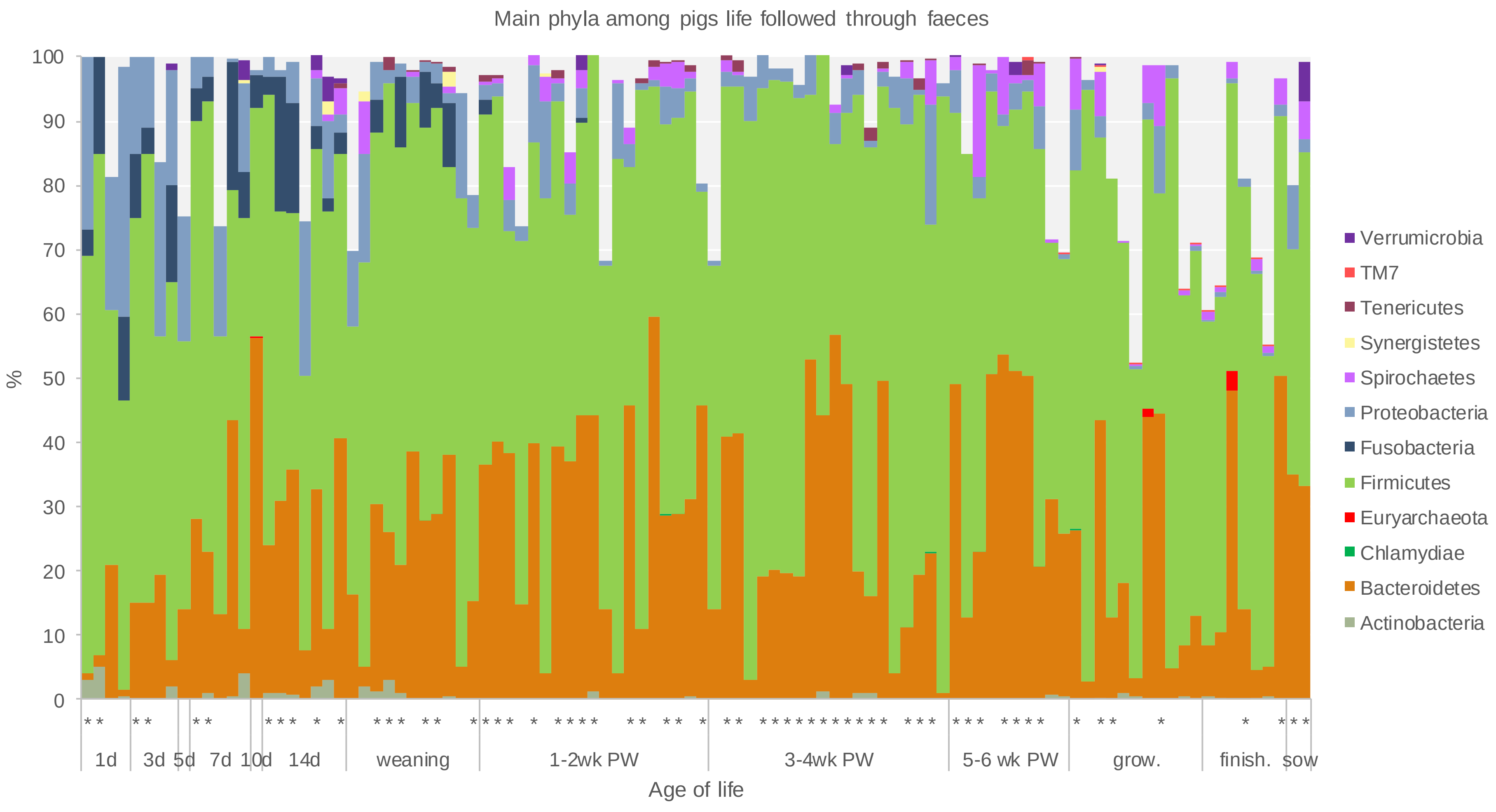


Figure 2.13 Evolution of the relative abundant (%) of the main phyla described over pig's life with the analysis of faecal samples. Profiles highlighted with '*' indicate measurements extracted from studies only displaying graphical information. Legend: 1d, 3d, 5d, 7d, 10d, and 14d are days of life; weaning are 21-28 days of life; wk PW are guiding post-weaning weeks; and grow. and finish. are growing (11-16 weeks old) and finishing (<19 weeks old) pigs, respectively. For detailed information see Appendix B.

2.4.2 Different microbial niches found along the GIT

We have seen in the previous section that, over pig's life, a colonisation sequence of successive microbial ecosystems is observed along the first weeks of life similar to that in the gut (from small intestine to colon) (De Rodas et al., 2018). Altogether, in the adult pig, it is also possible to find differences in the microbiota structure along the GIT. Significant variations in the physicochemical conditions and in the availability of growth substrates promote or hamper the colonisation of different niches by diverse species. For practical reasons, faeces are usually the samples of choice to study intestinal microbial communities. However, results show that faeces alone are not informative of the ecology of all intestinal habitats (Figure 2.14), and differences on specific locations could reveal other microbial targets that could be manipulated to influence gut health.

Stomach is an emerging important site of the GIT to be evaluated since it is the first barrier against external substances or microorganisms. Stomach has been characterised in weaned piglets with qualitative and quantitative differences related to distal parts. For instance, Motta et al. (2017) found that the major gastric phyla were Proteobacteria accompanied by Actinobacteria and Firmicutes, which also varied in terms of luminal-mucosa populations. Also, Newman et al. (2018) found Firmicutes and Proteobacteria the major representatives in gastric lumen, including Enterobacteriaceae and *Actinobacter* groups. Other works, however, have found a high prevalence and variety of *Lactobacillus* species (Mann et al., 2014; Mu et al., 2017). Interestingly, Mu et al. (2017) showed that stomach, along with duodenum, have more diverse communities compared to distal small intestine, suggesting that the advance of external bacteria would have been limited towards distal parts where other selective conditions are encountered. Stomach microbiota can be highly influenced by the diet that is consuming the animal. In this regard, as an example, in stomach *Herbiconiux* has been described as the major genus found, whih is related to plant degradation (Motta et al., 2017). But when evaluating mucosal communities after accurately rinsing the tissue during the sampling, this diversity of bacteria was still existent (Mann et al., 2014), even higher and differently structured to that of contents (Motta et al., 2017).

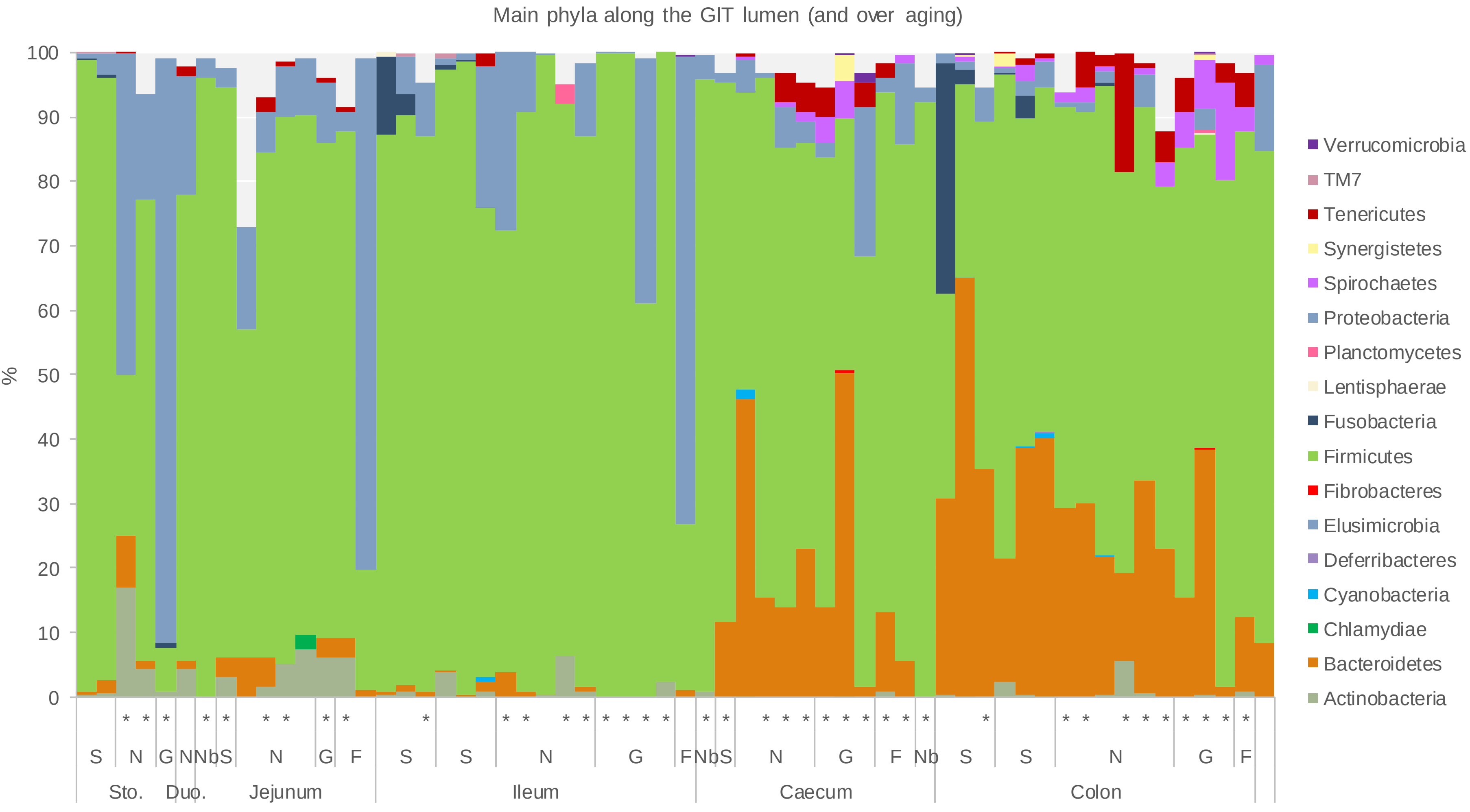


Figure 2.14 Relative abundance (%) of the main phyla found along the luminal GIT segments of the pig. Profiles highlighted with '*' indicate measurements extracted from studies only displaying graphical information. Legend: Nb are new-born piglets; S are suckling piglets; N are nursery piglets; G are growing pigs; and F are finishing pigs; and Sto. and Duo. refer to stomach and duodenum contents, respectively. For detailed information see Appendix B.

In adult pigs, small intestine communities are structured differently to those from the hindgut (Newman et al., 2018) and show to be more similar to that of stomach (Figure 2.14). Jejunum and ileum compartments also harbour mainly Proteobacteria and Firmicutes (Looft et al., 2014a; Zhao et al., 2015), including Enterobacteriaceae and *Escherichia*, *Acinetobacter*, and *Psychrobacter*, while in large bowel Clostridiales and Ruminococcaceae are the predominant groups (Zhao et al., 2015). A similar picture has been observed in the small intestine (ileum) of nursery pigs, represented mainly by Firmicutes, including *Lactobacillus*, and Proteobacteria, including *Escherichia*. Actually, several of the microbial groups that characterise the weaning transition, as for instance Actinobacteria, remain in the adult life (Zhang et al., 2018a). Large intestine (caecum and colon) communities are richer than in the foregut and resemble the faecal profile (Figure 2.14). Some of the characteristic groups also fall within those encountered during the weaning, like *Prevotella* (Bacteroidetes) altogether with *Megasphaera* (Veillonellaceae), and Lachnospiraceae, Ruminococcaceae families, and *Faecalibacterium* (Clostridiaceae).

These differences found along the gut were discussed by Roca et al. (2014) reflecting the physicochemical milieu and the digestive properties of each site. For instance, proximal tract might determine a more acidic environment, faster transit time and higher bile acid concentration as well as oxygen availability, whereas distal parts provide a more neutral pH, slowed intestinal transit and low oxidation-reduction potential that would allow higher rates of survival of bacteria. In this sense, in the small intestine functional profiles are correlated with bile acids, also related to amino acid, energy and lipid metabolisms (Zhang et al., 2018a), and related to survival mechanisms, e.g. dormancy and sporulation, associated to genera *Anaerobacter* and *Turicibacter* (Looft et al., 2014a). In the large intestine, enzymes for carbohydrate metabolism and related to fibre degradation are the predominant microbial functions (Looft et al., 2014a; Mu et al., 2017).

As it was mentioned before, there have been described differences between lumen and mucosa-associated microbial ecosystems. Zhang et al. (2018a) hypothesised that luminal microbiota may be more involved in the metabolism and digestion of nutrients, while the mucosal microbiome may be more involved in the immune function. In fact, several authors (De Rodas et al., 2018; Zhang et al., 2018a) observed that mucosa was a uniquely place for some families, mainly Proteobacteria (Enterobacteriaceae,

Campylobacteriaceae, Helicobacteriaceae, Desulfovibrionaceae); in counterpart, *Prevotella* was, rather, luminal. However, not all works showed, for instance, *Prevotella* and Bacteroidetes exclusive from lumen but also adhering to gut wall even at upper parts of the tract (Figure 2.15). Helicobacteriaceae and Campylobacteriaceae for instance have greater capability of colonising the mucin (Naughton et al., 2013), thus in closely proximity to immune cells that reside mucosa can act as stimulants (Cherourtre, 2005). Moreover, again the oxygen distribution between lumen and epithelium surface might have divided the populations with those obligate anaerobic bacteria abundant in the lumen distal parts, and those microaerophilic enriched in the mucosa (DeRodas et al., 2018; Zhang et al., 2018a). Mucosa, therefore, can harbour bacteria that are specialised to thrive in the mucosal environment, what would suggest a less diverse community than that in the lumen. However, Looft et al., (2014a) revealed high bacterial richness at mucosal sites compared to lumen, regardless of the gut site, with diversity and richness comparable to that of hindgut lumen (Figure 2.15).

Although numerous studies have profiled the GI microbiota of the pig, they focused on temporal sampling limited to faecal or unique GIT sites to define each age composition. Nonetheless, beyond temporal, anatomical differences have been shown throughout the entire GIT and between luminal and mucosal ecosystems. Although few works, similar dynamics and communities were resembled from the studies published up today. Therefore, depending on the scenario to be evaluated, for instance an intestinal disease, or the effects with specific dietary treatments, the assessment of the microbiota of the target site might be primordial and more helpful to understand the response.

Figure 2.15 Relative abundance (%) of the main phyla found along the mucosa of each GIT segment in the pig. Profiles highlighted with '*' indicate measurements extracted from studies only displaying graphical information. Legend: Nb are new-born piglets; S are suckling piglets; N are nursery piglets; G are growing pigs; and F are finishing pigs; and Sto. and Duo. refer to stomach and duodenum contents, respectively. For detailed information see Appendix B.

2.4.3 Gut dysbiosis around weaning

As described by several authors, the most dramatic change seen on gut microbiota is driven by weaning, which is de facto the change of diet. Actually, we can look at it as an abrupt dietary shift from exclusively sow milk intake to a complex solid diet that produces also an abrupt taxonomic and functional-related shift in the gut microbiota, rather than a transient state to the stable adult microbiota. In general terms, nonetheless, still a succession is likely to occur during suckling, with tendency of Bacteroidetes to expand and Proteobacteria to drop, that suddenly stops with weaning and *Prevotella* becomes the major genus during a relative short post-weaning time. The dysbiosis arisen from this dramatic change was formulated in the extensive review of Gresse et al. (2017) as a general decrease of the diversity with the underrepresentation of anaerobic bacteria such as Clostridia and Bacteroidia and the consequent rise of facultative anaerobes like Enterobacteriaceae, what can increase the risk of gastrointestinal diseases. It is worth mentioning that potential opportunistic pathogens, e.g. *Camylobacter*, Fusobacteria, and Clostridia, are detected at all GIT sites and represent a not negligible fraction (Mann et al., 2014). However, the dysbiosis and gastrointestinal infections have been poorly documented.

Although few, there are at least three studies evaluating the microbiota response of weaning piglets towards an ETEC F4 challenge, as well aiming to determine the earlier microbiota that may predict lately the clinical response (Table 2.5). The study of Bin et al. (2018) evaluated both jejunal and faecal microbiotas. Animals developing diarrhoea evidenced poorer diversity and altered community structure; however, further analysis did not enlighten which were the causative bacteria neither retrospectively nor de facto. Pollock et al. (2018) also observed an immediate reduction on faecal richness after the oral challenge and microbial composition differed when the pathogen was excreted at highest rates (day 8 post-infection), but neither differences could be detected on specific taxonomic groups. Similarly, Dou et al. (2017) tried to elucidate which microbial profile determined at an earlier age (7 days of life) would later predispose piglets to post-weaning diarrhoea. Diarrhoeic animals had higher Clostridiaceae, Enterococcaceae and Actinomycetaceae, including *Fusobacterium* and *Corynebacterium*, while healthy animals were characterised by higher Ruminococcaceae, Lactobacillaceae, Lachnospiraceae and

Prevotellaceae abundances (Table 2.5). In the same direction, immediately two days after the inoculation with *Salmonella* Typhimurium in weaners, members of Lachnospiraceae and Ruminococcaceae found in ileum also diminished, as well as *Lactobacillus* and *Clostridium*, among others (Agrüello et al., 2018). Other experiments focused on challenge models with *Salmonella* Typhimurium and *Lawsonia intracellularis* during post-weaning (Borewicz et al., 2015) were able to demonstrate divergence between infected animals and controls regardless of the course time, showing the greatest impact in colon rather than in small bowel (jejunum nor ileum). Both challenges decreased firstly Bacteroidetes followed sequentially by Spirochetes and finally Verrumicrobia. Also, specific changes were caused by each pathogen. In the *L. intracellularis* infection Actinobacteria and *Lactobacillus* were increased while Proteobacteria diminished; Fibrobacteres decreased after the *Salmonella* challenge. The same authors also traced earlier faecal microbiota from animals in commercial farms differentiating retrospectively high-*Salmonella* shedders from mid-low shedders. *Prevotella*, *Lactobacillus*, *Anaerobacter*, *Treponema*, *Roseburia* and *Campylobacter* were the genera depressed in the high-*Salmonella* shedders.

Table 2.5 Impact of different pathogen challenges inducing diarrhoeic processes on pig microbial populations evaluated through the HTS analysis of the 16S rRNA gene.

	Diarrhoeic diseases					
	Dou et al. (2017)	**Bin et al. (2018)**		**Pollock et al. (2018)**		
	Predisposed PWD	**ETEC F4**		**ETEC F4**		
	early life (7 days old)	nursery (18 days old)		nursery (4 weeks old)		
	faeces	jejunum	faeces	faeces		
↑	Clostridiaceae	F/B ratio	F/B ratio	None		
	Enterococcaceae	*Lactococcus*	*Escherichia-Shigella*			
	Actinomycetaceae	*Streptoccocus*				
	Corynebacterium	*Escherichia-Shigella*				
	Fusobacterium					
↓	Lactobacillaceae	*Weissella*	*Prevotella*	None		
	Lactobacillus					
	Lachnospiraceae					
	Ruminococcaceae					
	Prevotellaceae					
	Prevotella					
	Borewicz et al. (2015)			**Argüello et al. (2018)**		
	***Salmonella* Typhimurium**		***Lawsonia intracellularis***	***Salmonella* Typhimurium**		
	nursery (6 weeks follow-up)			nursery (5 weeks)		
	small bowel	colon	colon	ileum	ileum mucosa	faeces
↑	*Streptoccocus*	*Anaerobacter*	*Anaerobacter*	Mogibacteriaceae	Enterobacteriaceae	*Candidatus* arthromitus
	Prevotella	*Oribacterium*	*Lactobacillus*	Ruminococcaceae		*Odoribacter*
		Paraprevotella	*Oribacterium*			*Akkermansia*
			Paraprevotella			
			Actinobacteria			
↓		Bacteroidetes	Bacteroidetes	Clostridia	Clostridia	Clostridia
		Prevotella	*Prevotella*	Lachnospiraceae	*Lactobacillus*	*Clostridium*
		Spirochaetes	Proteobacteria	Ruminococcaceae	*Megasphaera*	*Dialister*
		Verrumicrobia	Fibrobacteres		*Mitsuokella*	*Lactobacillus*
			Spirochaetes			
	Legend:	Firmicutes	Bacteroidetes	Proteobacteria	Actinobacteria	Fibrobacteres
		Fusobacteria	Verrumicrobia			

2.4.4 Impact of antibiotics and their alternatives on GIT microbiota

Antimicrobials are frequently used in swine production for the purpose of growth promotion, disease prevention, or disease treatment. However, their usage in pigs may help to maintain a reservoir of antimicrobial-resistant bacteria and resistance genes (ARG) (Xiao et al., 2016). The development of alternatives to antimicrobials in swine requires a better understanding of how the microbiota is affected by these agents. The impact of antibiotic administration on microbiota has been widely studied but sometimes with inconclusive results. On one hand, with the comprehensive sampling of Xiao et al. (2016), the use of antibiotics in feed could be associated to the reduction of faecal richness. This reduction of ecosystem biodiversity induced by antibiotics has been also described by other authors (Looft et al., 2014b). However, identifying common changes in particular microbial groups related to the use of antimicrobials has not been possible. In this regard, the so-called meta-analysis of Holman et al. (2017), compiling raw data from multiple studies, did not find any unanimous trend for antibiotics impact on microbiota. The absence of univocal effects of antibiotics on microbiota composition would be somehow expected considering multiple factors such as the type of antibiotic, their mechanism of action, the dose or the environmental challenge. These diverging results can be seen also in Table 2.6 with different changes in microbial groups for each of the particular works.

Table 2.6 Impact of different in-feed antibiotics on GIT pig microbial populations evaluated through the HTS analysis of the 16S rRNA.

	Antibiotics						
	Looft et al. (2014a) **ASP250** ?? days (3 months old) grower		**Looft et al. (2014b)** **Carbadox** 4 days nursery	**Mu et al. (2017)** **Oxytetracycline + olaquindox + kitasamycin** 35 days early life to nursery (7 to 42 days old)			
	caecum	colon	faeces	stomach	duodenum	jejunum	colon
↑	*Anaeroplasma*	*Lachnobacterium*	*Asteroleplasma*			Proteobacteria	*Treponema*
	Lachnobacterium	*Salsuginibacillus*	*Faecalibacterium*			*Escherichia*	
	Salsuginibacillus	*Escherichia*	*Roseburia*			*Actinobacillus*	
	Paraprevotella		Bacteroidetes				
	Eschericicha		*Prevotella*				
↓	*Streptococcus*	*Parasporobacterium*	Firmicutes	*Clostridium*	*Bacillus*	*Clostridium*	Tenericutes
	Helicobacter	*Streptococcus*	*Lactobacillus*	*Bacillus*	*Clostridium*	*Sharpea*	*Prevotella*
	Treponema	*Turicibacter*	Proteobacteria	*Sharpea*	*Sharpea*	*Bacillus*	
		Treponema	Elusimicrobia	Bacteroidetes			
			Planctomycetes	*Prevotella*			
			Lenthisphaerae	Tenericutes			
	Li et al. (2018) **Bacitracin + colistin + olaquindox** 42 days nursery colon	**Soler et al. (2018)** **Amoxycilin + colistin** 30 days nursery faeces	**Poulsen et al. (2018)** **Gentamicin** 3 days early life ileum				
↑	*Escherichia-Shigella*	Bacteroidetes					
		Prevotellaceae					
↓	Cyanobacteria	Firmicutes	*Sarcina*				
	Verrumicrobia						
	Legend:	Firmicutes	Bacteroidetes	Proteobacteria	Actinobacteria	Cyanobacteria	Elusimicrobia
		Lenthisphaerae	Planctomycetes	Spirochaetes	Tenericutes	Verrumicrobia	

Not necessarily different populations affected by antibiotics were the same as those altered after weaning or under a dysbiosis. For instance, in the works of Soler et al. (2018) and Poulsen et al. (2018), younger animals (suckling and nursery piglets) presented scarce effects with the administration of the antibiotic. Trying to get some insights, Mu et al. (2017) hypothesised that the effects were located on the foregut (stomach to jejunum) rather than distal tract. Li et al. (2018), in counterpart, were able to detect differences in colon with the supplementation of a mixture of antibiotics on microbial structure being alpha diversity reduced with decreases in the phyla Cyanobacteria and Verrumicrobia concomitant with increases in the *Escherichia-Shigella* group. Looft et al. (2014a) supplementing ASP250 were able to discriminate communities despite total richness was the same. Some potential pathogens were reduced (*Treponema*, *Streptococcus*, *Helicobacter*, and *Turicibacter*) but *Escherichia* increased. Also, Looft et al. (2014b), in another study, found that supplementing carbadox to nursery piglets for one week, but not further, differentiated medicated from non-medicated animals and reduced richness.

Several strategies are being continuously evaluated to improve gut health in livestock, trying to limit the use extent of antibiotics. Despite this, few studies address their possible impact on pig intestinal microbiota. Between those strategies most studied, we find probiotics and organic acids (Table 2.7). Although probiotics have been deeply studied in swine as in humans (Turner et al., 2001; Gaggìa et al., 2010; Liu et al., 2018), scarce works have been published related to their impact on the gut microbiota of the pig. Common probiotic strains such as *Bacillus* spp. spores have been shown to have little impact on young piglet microbiota (Poulsen et al., 2018), while *Lactobacillus rhamnosus* has been shown to exert a modulatory effect on faecal populations of weanlings in combination with cocoa powder (Solano-Aguilar et al., 2018). Xu et al. (2018c) also supplementing two types of yeast-probiotics based on *Saccharomyces cerevisae* (*S. cerevisae* fermented malt grown with or without egg white) observed an impact on animal performance and microbiota adaptation. *Clostridium butyricum* was also supplemented in weaners (Zhang et al., 2018b) with improvements in growth but changes only in minor faecal microbial groups. Probiotics are continuously of interest because of their positive impact on the livestock performance, however, although as direct-fed microbials would be expected to

affect to a greater extent the microbiome, the scarce studies published in pigs and the diversity of factors implied in each design do not allow any conclusions.

Regarding the use of organic acids, and particularly short- and medium-chain fatty acids, despite the high number of studies testing their potential as antibiotic replacers (reviewed in the first part of this literature review), only few works have evaluated comprehensively their effect on the gut microbiota (Table 2.7). Moreover, the few works found in literature show very dissimilar results, with no evidence of general trends to promote, e.g. beneficious, or inhibit, e.g. pathogenic, specific populations. As stated above, it should not be discarded possible effects of these acids driven towards upper parts of the tract that only have been object of study in a reduced number of works. In this regard, with the supplementation of sodium butyrate in new-born piglets, Xu et al. (2016) observed that gastric populations were principally those affected. Other combinations of MCFA and organic acids (OA) tested in the works of Soler et al. (2018) or Li et al. (2018) also showed the same patterns but with fewer modifications. Nonetheless, the use of formic acid, at increasing doses, showed major reductions of potential pathogens like *Fusobacterium*, *Turicibacter* or *Streptococcus* in jejunum, what would suggest a more prominent antimicrobial effect as has been reviewed in this Thesis for this acid. Despite these changes, authors could not find reductions (nor changes) in richness (Luise et al., 2017) like usually was found after the use of some antibiotics.

Table 2.7 Impact of different antibiotic alternatives (probiotics and organic acids) on pig GIT microbial populations evaluated through the HTS analysis of the 16S rRNA.

Probiotics					
	Poulsen et al. (2018) ***Bacillus* spores** mother & piglet colon	**Solano-Aguilar et al. (2018)** ***L. rhamnosus* + cocoa powder** 28 days nursery faeces	**Xu et al. (2018c)** ***S. cerevisae* malt** 7 days pre- & post-weaning faeces	 ***S. cerevisae* egg white** 7 days faeces	**Zhang et al. (2018b)** ***C. butyricum*** 28 days nursery faeces
↑	Fusobacteria	Anaeroplasmataceae	*Coprococcus*	*Anaeroplasma*	*Acidaminococcus*
	Fusobacterium	Lachnospiraceae	*Dorea*	Clostridia	*Megasphaera*
		Ruminococcaceae	*Enterococcus*	*Dorea*	*Mitsuokella*
		Clostridium incertae sedis XIV	*Holdemania*	*Peptococcus*	Selenomonadales
		Bacteroidaceae	Bacteroidetes	*Ruminococcus*	*Selenomonas_3*
		Porphyromonadaceae	*Bacteroides*	*Enterococcus*	*Veillonella*
		Rikenellaceae	YRC22	*Holdemania*	*Prevotella_7*
		Sphingobacteriaceae		*Sharpea*	*Sutterella*
	Bacteroidetes	Desulfovibrionaceae		*Campylobacter*	
	Bacteroides	*Succinivibrio*		*Desulfovibrio*	
	YRC22	Spirochaetaceae		*Succinivibrio*	
↓		F/B ratio	Firmicutes		*Anaeroplasma*
		Clostridiales			Clostridiales
		Blautia			Lachnospiracae
		Faecalibacterium			*Coprococcus_1*
		Lactobacillus			*Dialister*
		Streptococcus			*Lactobacillus*
					Helicobacter
	Legend:	Firmicutes	Bacteroidetes	Proteobacteria	Actinobacteria
		Fusobacteria	Spirochaetes	Verrumicrobia	Cyanobacteria

Table 2.7 (continued).

Organic acids						
	Xu et al. (2016) **Sodium butyrate** 7 days early life stomach	 colon	**Luise et al. (2017)** **Formic 1.4 %** 49 days nursery jejunum	 **Formic 6.4 %** 49 days jejunum	**Li et al. (2018)** **OA blend** 49 days nursery colon	**Soler et al. (2018)** **OA blend** 30 days nursery faeces
↑	Aerococcaceae	*Faecalibacterium*		*Streptococcus*	*Prevotella*	*Bacillus*
	Facklamia	*Roseburia*			Tenericutes	*Lactobacillus*
	Globicatella	*Subdoligranulum*				
	Flavobacteriaceae	*Odoribacter*				
	Uncl. Prevotellaceae	*Corynebacterium*				
	Acinetobacter					
	Actinobacillus					
	Pastereullaceae					
	Uncl. Neisseriaceae					
	Micrococcaceae					
	Kocuria					
	Rothia					
	Uncl. Leptotrichiaceae					
↓	Lactobacillaceae		*Gemella*	*Gemella*	Cyanobacteria	
	Lactobacillus		*Lactobacillus*	*Lactobacillus*	Verrumicrobia	
			Parvimonas	*Leuconostoc*		
			Streptococcus	*Parvimonas*		
			Turicibacter	*Acinetobacter*		
				Fusobacterium		
	Legend:	Firmicutes	Bacteroidetes	Proteobacteria	Actinobacteria	Cyanobacteria
		Fusobacteria	Spirochaetes	Verrumicrobia		

In summary, the establishment of the pig gastrointestinal microbiota is a complex and successional process with quantitative and qualitative changes that initialises immediately at birth. In commercial conditions, nonetheless, a dramatic disruption occurs due to weaning stresses and potential pathogens commonly found more opportunities to arise. A cross-talk relation exists among the host and microbiota. Different studies have demonstrated how bacteria are adapted to specialised niches along the gut compartmentalisation, whereby the host also exerts selective pressure determining which communities cohabite and finally are vital for the animal health. From this point of view, it seems essential to define the reference composition of the healthy microbiota and get knowledge on its role in improving the growth performance and the control of enteric bacterial diseases. This knowledge will open the window to understand the exact mechanisms of action of antibiotics and will help in the development of alternatives such as short- and medium-chain fatty acid-based additives. Further insights must be done to decipher the complexity of the responses among these additives. As a quick developing technology, next generation sequencing will provide more accurate results and help in their interpretation to support or even overcome the bulk of information generated until now.

Chapter 3 Objectives and experimental design

This PhD dissertation is part of the project PORCDIGEST (ref. IDI-20140262) funded by the Centro para el Desarrollo Tecnológico Industrial (CDTI) and NOREL S.A. This project is framed within the research line of our group based on the study of different feeding strategies aimed to improve the health of young pigs around weaning, with special attention to prevent and control intestinal pathogens, trying to meet current production requirements on restrictions to antibiotic use.

The main hypotheses of the present PhD dissertation are:

1. Feed additives based on monocarboxylic fatty acids consisting of short- and medium-chains may be beneficial on the control and prevention of intestinal pathogens and the promotion of the gut health in weaning piglets.
2. The combination of these different monocarboxylic fatty acids as new functional protected forms may exert synergistic effects on the gut health of the animals.

The main objectives of the PhD dissertation are, therefore the following:

1. To evaluate the potential of new short- and medium-chain fatty acids forms as in-feed additives to fight digestive pathogens in weaning pigs. New forms consist of: (i) two new protected forms of sodium butyrate or sodium heptanoate, (ii) a mixture of sodium salts of distillates of coconut medium-chain fatty acids (MCFA), or (iii) sodium butyrate or sodium heptanoate protected with the sodium salts of distillates of coconut MCFA.
2. A secondary objective of this Thesis aimed to further investigate the intestinal microbiota structure of weaning pigs analysing the impact of these additives by the high-throughput sequencing (HTS) of the 16S rRNA gene.

In order to accomplish these objectives, four experiments were performed under the same experimental design and were included in a scaling sequence along Chapters 4 to 6.

In **Chapter 4**, two new forms of protected sodium butyrate and protected sodium heptanoate were evaluated in weaned piglets experimentally challenged with enterotoxigenic *Escherichia coli* (ETEC) F4. In this trial, the main variables assessed were animal performance, clinical signs, pathogen quantification, fermentation profile,

immune response, intestinal morphology, and microbiota profiling by HTS of 16S rRNA gene sequencing.

In **Chapter 5**, a mixture of sodium salts of distillates of coconut MCFA was evaluated in two different trials. The first trial was performed with weaned piglets experimentally challenged with *Salmonella* Typhimurium, and the second trial with ETEC F4. The same variables as in Chapter 4 were assessed.

In **Chapter 6**, the mixture of sodium salts of distillates of coconut MCFA protecting either sodium butyrate or sodium heptanoate were evaluated in two different trials, both consisting of weaned piglets experimentally challenged with ETEC F4. The same variables as in Chapter 4 were assessed.

Chapter 4 Response of gastrointestinal fermentative activity and colonic microbiota to protected sodium butyrate and protected sodium heptanoate in weaned piglets challenged with ETEC F4

4.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the major pathogen responsible for post-weaning diarrhoea in pigs (Fairbrother et al., 2005). This is an opportunistic disease, also called colibacillosis, which takes advantage of unfavourable conditions around weaning, allowing pathogenic serotypes to become dominant (Dunne, 1959). This disorder represents important economic losses for the pig industry due to depression of growth and performance of weanlings, being one of the principal causes of mortality (Heo et al., 2013). It is estimated that up to 66 % of ETEC colonised nursery pigs can remain subclinical but with a compromised welfare and productivity. Moreover, up to 95 % of the isolated strains of ETEC have been reported to be resistant to at least one antimicrobial (Moredo et al., 2015), and furthermore EFSA reported a high presence of antibiotic multi-resistant (AMR) strains of *E. coli* in fattening pigs in 2015 (EFSA & ECDC, 2017).

Acidifying the feed of weanling piglets is an old nutritional practical handling (Edmonds et al., 1985) that recently gained special interest since the ban of the use of antibiotics as growth promoters (AGP) in the European Union in 2006 (R(CE) 1831/2003). In this regard, organic acids have been purposed as alternatives to antibiotics to be used in piglet diets since they are known to be helpful in overcoming problems occurring in the post-weaning period (Tsiloyannis et al., 2001) and improving animal growth (Partanen & Mroz, 1999). Several mechanisms of action have been described underlying these effects, being directed to the host or to gastrointestinal microbiota (Bergman, 1990). For the host, organic acids can be a source of energy (Dierick et al., 2003; Gaudier et al., 2004) or can reduce the inflammatory response (Jiang et al., 2015). Among their effects on the microbiota, they have demonstrated a modulatory effect by promoting beneficious populations (Gálfi & Bokori, 1990; Hanczakowska, 2017) or by reducing enteric pathogens (Hansen et al., 2007; Sun & O'Riordan, 2013). Moreover, organic acids have shown to induce the expression of host defence peptides (HDP) against pathogens (Sunkara et al., 2012) finally triggering an interaction between both host and microbiota. For this reason, understanding their action is going one step beyond the acidification since their activity on the gut is more complex and depends on the concentration, time of administration or

their nature, i.e. the length of the carbon chain or the degree of saturation (Cherrington et al., 1991; Mroz et al., 2006).

Focusing on short-chain fatty acids (SCFA), and more concretely butyric acid, or its sodium salt, beneficial effects have been proved in the same way on performance of piglets around weaning (Lu et al., 2008), on the modulation of the intestinal microbiota (Castillo et al., 2006; Xu et al., 2016) and on the stimulation of the immune response (Melo et al., 2016). In the case of medium-chain fatty acids (MCFA), they have also shown improvements in productive yields of pigs (Dierick et al., 2004) with evidenced impact on gastric and intestinal microbiota (Zentek et al., 2012; Zentek et al., 2013). Especially heptanoic acid has been demonstrated *in vitro* to have clear bactericide properties (Bhattarai et al., 2007; Grilli et al., 2013).

Within this context, the present study aimed to evaluate the efficacy of two protected sodium salts of butyric and heptanoic acids to improve gastrointestinal health and fight post-weaning colibacillosis in piglets and elucidate possible mechanisms of action. To achieve this objective, an experimental model of disease by orally challenging piglets with F4 ETEC was used.

4.2 Material and methods

The experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona (UAB) and received prior approval (permit no. CEAAH 1619) from the Animal and Human Experimental Ethical Committee of this Institution. The treatment, management, housing, husbandry and slaughtering conditions conformed to European Union Guidelines (Directive 2010/63/EU).

Animals, housing and experimental design

The trial was conducted as a Level 2–High Risk Biosecurity Procedure with appropriate training of the personnel involved. A total of 72 male piglets [Large White x Landrace] x Piétrain weaned at 21 days of age and 5.4 ± 0.73 kg body weight (BW) were selected from

a commercial farm, where mothers did not receive any vaccination against *Escherichia coli,* and transported to the UAB facilities.

Pigs were maintained under a 13 h light/11 h dark lighting regimen. Each pen (3 m^2) had a feeder and a water nipple to provide food and water for *ad libitum* consumption. The weaning rooms were equipped with automatic heating and forced ventilation and an individual heat-light per pen. The experiment was conducted during the winter season (November – December), with an average inside room temperature of 28 ± 2 ºC.

Animals were distributed in three rooms of eight pens each (24 pens, 3 animals per pen) considering initial BW for a similar average BW within pens. The experimental treatments were distributed evenly among the three rooms with 8 replicates each.

Experimental products and diets

All feed additives were supplied by Norel S.A (Madrid, Spain) consisting of two salts of organic acids, sodium butyrate (Gustor BP70) or sodium heptanoate (Hept'on), protected: 70 % of active ingredient and 30 % of vegetable fat.

Diets (Table 4.1) were formulated to satisfy the nutrient requirement standards for pigs (NRC, 2012). All diets were manufactured in the same batch, and treatments were included in a second mixture, following the manufacturer's recommended dosages. The three experimental diets were as following: a plain diet without additives (CTR), the same diet supplemented with 3 kg/t of Gustor BP70 (BUT) (equivalent to 2.1 g of protected sodium butyrate/kg of feed); or with 3 kg/t of Hept'on (HPT) (equivalent to 2.1 g of protected sodium heptanoate/kg of feed).

Bacterial strain

The bacterial strain used in the present study was an F4 enterotoxigenic *Escherichia coli* (ETEC) strain (positive for virulence factors F4ab, F4ac, LT, STb and EAST1 and negative for EAST1, F6, F18, F41, STa, VT1, VT2 and EAE), isolated from faeces of 14-week pigs and provided by the Veterinary Laboratory for the Diagnosis of Infectious Diseases of UAB

(reference COLI30/14-3). The oral inocula were prepared by an overnight incubation at 37 ºC and 250 rpm in brain heart infusion (BHI) broth (Oxoid). Animals were orally inoculated on two consecutive days. On the first day, the pathogen was given directly in the growth medium at a dose of 9.6 x 10^8 CFU/mL. For the second day, bacteria were concentrated by centrifuging (2000 x *g*, 10 min and 4 ºC) and resuspending in 1/10 initial volume the original culture (final concentration 1.1 x 10^{10} CFU/mL).

Table 4.1 Ingredient composition and nutrient analysis of the basal diet as-fed basis (g/kg).

Ingredients			
Maize	280.7	Mono-calcium phosphate	21.3
Wheat	170.0	Calcium carbonate ($CaCO_3$)	8.2
Barley 2 row	150.0	L-Lysine HCL	4.5
Extruded soybean	122.4	Vitamin-Mineral Premix[A]	4.0
Sweet whey-powder (cattle)	100.0	Sodium chloride (marine salt)	3.0
Fishmeal LT	50.0	DL-Methionine 99	2.4
Soybean meal 44	50.0	L-Threonine	2.3
Whey powder 50 % fat	30.3	L-Tryptophan	0.9
Calculated nutrients			
Metabolisable energy (kcal/kg)	3315.4	Digestible lysine	12.5
Crude protein	187.7	Digestible methionine plus cysteine	7.5
Calcium	10.0	Digestible threonine	8.1
Digestible phosphate	6.0	Digestible tryptophan	2.8
Analysed nutrients			
Dry matter	912.3	Crude protein	173.4
Ash	67.7	Neutral detergent fibre	77.6
Crude fat	42.3	Acid detergent fibre	24.0

[A] Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D3, 39.9 mg vitamin E, 3 mg vitamin K3, 2 mg vitamin B1, 2.3 mg vitamin B2, 3 mg vitamin B6, 0.025 mg vitamin B12, 20 mg calcium pantothenate, 60 mg nicotinic acid, 0.1 mg biotin, 0.5 mg folic acid, 150 mg Fe, 156 mg Cu, 0.5 mg Co, 120 mg Zn, 49.8 mg Mn, 2 mg I, 0.3 mg Se.

Experimental procedure

The duration of the study was 16 days, in which performance and clinical parameters were evaluated. At arrival, faecal samples for microbiological analysis were obtained aseptically from the pig of medium weight of each pen. Live weight was recorded on days 1, 8, 12 and 16, while feed consumption was recorded on days 1, 7, 8, 9, 10, 11, 12, 13 and 16. The average daily gain (ADG), and average daily feed intake (ADFI) were calculated per pen.

After one week of adaptation, faecal samples were taken again for microbiological analysis, and thereafter two single 6 mL oral doses (5.8 x 10^9 CFU and 6.6 x 10^{10} CFU) of F4 ETEC were administered on consecutive days (8 and 9) to the animals. The second oral challenge was administered after confirming no clinical signs (fever or diarrhoea) 24 h after first oral inoculation. To ensure that the stomach was full at the time of inoculation and facilitate bacterial colonisation, feed withdrawal was done at 21:00 of the previous day and provided back 30 minutes before inoculation.

After the oral challenge, animals were checked daily for clinical signs to evaluate their status (i.e. dehydration, apathy and faecal score) always by the same person. Faecal score was measured on days 1, 2, 3, 5 and 8 post-inoculation (PI) using a scale: 0 = hard, 1 = solid and cloddy, 2 = soft with shape, 3 = very soft or viscous liquid and 4 = watery or with blood. Rectal temperature was assessed with a digital thermometer (Thermoval Rapid, Hartmann) on days 1 and 2 PI. Mortality rate was also registered, and no antibiotic treatment was administered to any of the animals along the experiment.

On the day of inoculation (0 PI), faecal samples for microbiological analysis were taken aseptically from the pig of medium weight from each pen (N = 24) after spontaneous defecation associated with the manipulation of the animal or by digital stimulation.

At days 4 and 8 PI, one pig per pen was euthanised to take blood, digesta and tissue samples. Serial euthanasia and sampling were performed during the morning between 8:00 and 14:00. On day 4 PI, the animal from each pen with the medium weight at the beginning of the study was euthanised. On day 8 PI, the heaviest one was euthanised. The rest of the animals were euthanised at the end of the trial. Prior to euthanasia, a 10-mL sample of blood was obtained by venepuncture of the cranial vena cava using 10-mL tubes without anticoagulant (Aquisel), and immediately animals received an intravenous lethal injection of sodium pentobarbital (200 mg/kg BW; Dolethal, Vetoquinol S.A). Once dead, animals were bled, the abdomen was immediately opened, and the complete gastrointestinal tract excised.

Digesta samples from stomach, duodenum, ileum and proximal colon (10 first cm from caeco-colic junction) were collected, homogenised, and a sample of 10 g was kept immediately on ice until being stored at -20 ºC for further analysis of short chain fatty

acids (SCFA), heptanoic and lactic acid. The pH of the ileum and colon contents was determined, after homogenisation of digesta, with a pH-meter (Crison 52-32 electrode, Net Interlab), calibrated on each day of use. Aliquots of 5 g from ileal and colonic samples were also kept on ice for microbiological analysis being processed in less than 4 h, and other similar aliquots were stored at -80 ºC for further *E. coli* F4 quantification by qPCR and high-throughput sequencing (HTS) of microbiota from colon. A set of ileal and colonic digesta samples was preserved in a H_2SO_4 solution (3 mL of digesta plus 3 mL of 0.2 M H_2SO_4) and kept frozen at -20 ºC until ammonia (NH_3) determination.

With the aim of analysing bacteria attached to the ileal mucus and epithelium, 15-cm long sections of ileum were excised, washed thoroughly three times with sterile PBS, opened longitudinally and scraped with a microscopy glass slide to obtain the samples to be analysed. One aliquot was kept on ice for microbiological analysis and processed in less than 4 h and a second aliquot immediately kept on ice and stored at -80 ºC for *E. coli* F4 quantification by real-time (quantitative PCR [qPCR]).

For the histological study, 3-cm long sections from the ileum were also removed, opened longitudinally, washed thoroughly with sterile PBS and fixed by immersion in a formaldehyde solution 4 % (Carlo-Erba Reagents).

Blood samples were centrifuged (1500 x *g* for 15 min at 4 ºC) after 4 h refrigeration; serum obtained was divided in different aliquots and stored at -20 ºC.

Analytical procedures

Chemical analyses of the diets including dry matter (DM), ash, crude protein and diethyl ether extract, were performed according to Association of Official Agricultural Chemists standard procedures (AOAC, 2007). Neutral-detergent fibre and acid-detergent fibre were determined according to the method of Van Soest et al. (1991).

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned at 4-µm thickness and stained with haematoxylin and eosin. Morphological measurements of ten different villus-crypt pairs were performed with a light microscope (BHS, Olympus) using the technique described in Nofrarías et al. (2006).

Serum concentrations of tumour-necrosis factor-α (TNF-α) were determined by Quantikine Porcine TNF-α kits (R&D Systems), and pig-major acute-phase protein (Pig-MAP) concentration was determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A), as described in Saco et al. (2011).

Ammonia (NH_3) concentration was determined with the aid of a gas-sensitive electrode (Hach Co.) combined with a digital voltmeter (Crison GLP 22, Crison Instruments S.A) modified from Barba-Vidal et al. (2017b). Three grams of digesta preserved in H_2SO_4 (1:2) were centrifuged at 1372 x *g* for 10 minutes. Supernatant was obtained and finally neutralised with 1 mL of 10 M NaOH to reach pH 11 while stirring and measuring ammonia released as different voltage in mV. Short-chain fatty acid (SCFA), heptanoate and lactic acid were determined based on the method of Richardson et al. (1989) modified by Jensen et al. (1995) performed by gas chromatography after submitting the samples to an acid-base treatment followed by a diethyl ether extraction and derivatisation with *N*-(tertbutyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MBTSTFA) plus 1 % *tert*-butyldimethylchlorosilane (TBDMCS) agent.

For microbial counts in faeces and ileal and colonic digesta, samples were 10-fold serial diluted in Lactated Ringer's solution (Sigma-Aldrich) and seeded in MacConkey agar and chromogenic agar (Oxoid) for the counting of enterobacteria and coliform, respectively. Counts were read after 24 h incubation at 37 ºC.

DNA from samples (approximately 250 mg) was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen), and subsequently its concentration and purity checked using NanoDrop 1000 Spectrophotometer (Thermo Fisher). The protocol was followed applying all recommended optimisation steps to improve bacterial cell rupture and purity. The DNA was finally eluted in 200 mL of Qiagen buffer AE and stored at -80 ºC until use for *E. coli* F4 quantification or microbiota HTS.

The pathogen was quantified in colonic digesta and ileal scrapings by qPCR using SYBR green dye. A qPCR targeting the gene coding the F4 fimbria of ETEC F4 was performed by modifying the procedure described by Gustavo Hermes et al. (2013). Changes consisted in using SYBR Green PCR Master Mix PCR (Applied Biosystems) and reducing the total volume of reaction to 20 µL, which included 10 µL of 2X SYBR Green PCR Master Mix PCR

buffer, 0.88 µL of each primer (12.5 µM) and 5 µL of DNA sample. The calculated limit of quantification was 3.83-log F4 gene copies/g fresh matter (FM).

The HTS was performed on colonic microbiota by targeting the V3-V4 region of 16S rRNA, using the kit MiSeq® Reagent Kit v2 (500 cycle) (MiSeq from Illumina). Primers used in the construction of libraries with amplicons of putative 460 bp were the following:

F-5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

R-5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Sequence reads of 16S rRNA gene generated from MiSeq Illumina® system were processed on QIIME v.1.9.1 pipeline (Caporaso et al., 2010) with default settings. The quality filter of already demultiplexed sequences was performed at a maximum unacceptable Phred quality score of Q20. Resulting reads were clustered to operational taxonomic units (OTUs) using uclust with 97 % sequence similarity and subsampling pick open reference method (Rideout et al., 2014) at 10 % of sequences subsampled. Representative sequences were assigned to taxonomy against bacterial 16S GreenGenes v.13.8 reference database (DeSantis et al., 2006) at a 90 % confidence threshold, and sequence alignment and phylogenetic tree building were obtained through uclust and FastTree. Thereafter, chimeric sequences were removed with ChimeraSlayer (Haas et al., 2011) with default settings, and further quality filtering consisted in removing singletons and OTUs with relative abundance across all samples below 0.005 % as recommended by Bokulich et al. (2013).

Statistical analysis

The effect of experimental treatments on performance, microbial (log transformed for analysis) and slaughter measurements was performed in free software R v1.9 using the stats package (R Core Team, 2013) lm() function for two-way ANOVA including the treatment effect (diet) and block effect (room). When the block effect was not observed (P >0.05), one-way ANOVA was performed only with treatment effect. Average daily feed intake, rectal temperature and daily faecal consistency were performed using the lme4

package (Bates et al., 2015) lmer() function for adjusted linear mixed model with a period-by-treatment interaction term.

For all analysed data, the pen was used as the experimental unit. The alpha level for the determination of significance for all the analysis was 0.05. The statistical trend was also considered for *P* values >0.05 and <0.10. Data are presented as means and residual standard error (RSE). When treatment effect was established (*P* <0.05), multcomp package (Hothorn et al., 2008) glht() function was used to make multiple comparisons under Dunnett method with CTR group as control. When day effect was established (*P* <0.05), Tukey adjustment was used.

Biostatistics of quality-filtered sequences were performed in open source software R v.3.4.3. Firstly, OTU table was imported to R with phyloseq package (McMurdie & Holmes, 2013). Only OTUs shared among experimental groups were included and unique OTUs excluded. Diversity was analysed at OTU level using vegan package (Oksanen, 2017). Richness and alpha diversity were calculated with raw counts based on Chao1 estimator (Chao, 1984) to estimate the number of taxa in the community and Shannon index (Shannon, 1963) that considers the richness as well as the evenness. For beta diversity, Whittaker distance matrix was calculated based on relative abundances with betadiver() function. To compare any differential effects from treatments, an ANOVA was performed for richness and diversity. Finally, differential abundance analysis was performed with the OTU and taxa relative abundances because of the compositional nature (Gloor et al., 2017) under a zero-altered negative binomial or negative binomial model with pscl (Zeileis et al., 2008) and mass packages (Venables & Ripley, 2002), respectively, and corrected by false-discovery rate (FDR) (Benjamini & Hochberg, 1995).

4.3 Results

In general, the trial proceeded as expected. The oral challenge promoted a mild course of diarrhoea, from which the animals recovered spontaneously without pharmacological support. No animal received veterinary treatment along the study. The mortality did not reach 5 % with only three deaths registered (excluding euthanised animals). On the first week of adaptation, one animal from HPT group presented acute inflammation in one leg

and was euthanised for ethical reasons. A second pig from BUT group was also euthanised on day 5 PI following the clinical chart guide, and a third animal from BUT group was found dead on day 8 PI. Casualties came from different pens.

Animal performance

Performance in terms of BW, ADG and ADFI is presented in Table 4.2. During the first week (before the challenge), ADG was registered very low and close to 0 with a high residual variation for the three experimental diets (2.6 ± 46.60 g/day) reflecting a marked post-weaning syndrome in some pens. Regarding the effect of experimental diets on performance, no significant differences were registered in final BW, ADFI or ADG in neither pre- nor post-inoculation period.

Table 4.2 Effect of the experimental treatments on growth performance.

	Treatments				
	CTR	**BUT**	**HPT**	**RSE**	***P***
BW (kg)					
Initial	5.38	5.40	5.39	0.100	0.848
Final	7.08	7.57	7.42	1.336	0.755
ADFI (g/day)					
Pre-inoculation^{A}	105	125	100	49.8	0.717
Post-inoculation^{B}	316	385	358	138.9	0.609
ADG (g/day)					
Pre-inoculation^{A}	2.05	5.66	0.04	48.7	0.973
Post-inoculation^{B}	218	226	233	119.9	0.968

^{A} Pre-inoculation: Period from 0 – 7th experimental day. ^{B} Post-inoculation: Period from 8 – 16th experimental day. Legend: BW, body weight; ADFI, average daily feed intake; and ADG, average daily gain.

Clinical signs

The evolution of the faecal consistency of the animals along the post-inoculation (PI) period is exposed in Figure 4.1. After the oral challenge (1 PI), faecal consistency worsened in all experimental groups (*P* <0.001) and recovered on days 5 and 7 PI with no differences between diets (*P* = 0.533). Regarding rectal temperature, it decreased the day after the challenge from 39.4 ± 0.29 ºC on day 0 PI to 38.7 ± 0.07 ºC on day 1 PI and 38.7 ± 0.24 ºC on day 2 PI (*P* <0.001). No differences were found between diets (*P* = 0.30).

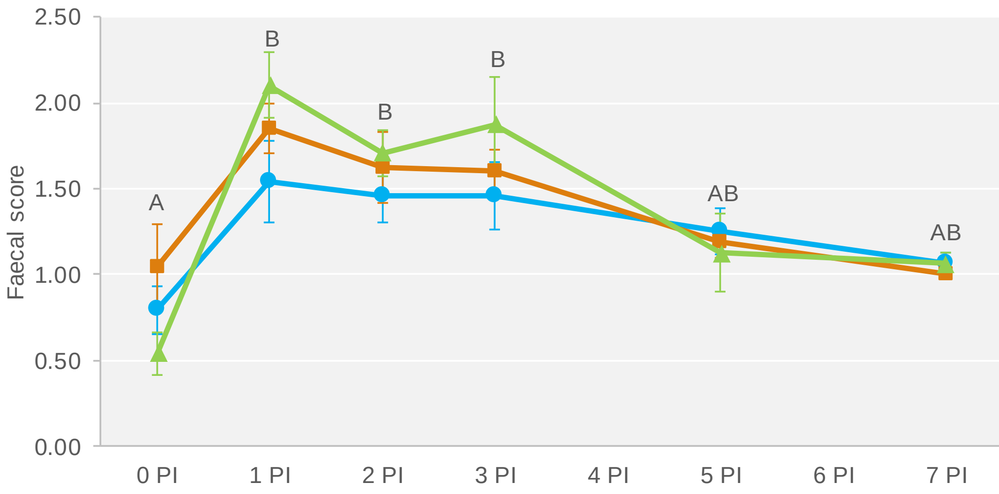


Figure 4.1 Evolution of mean faecal scores in the different experimental groups CTR (●), BUT (■), and HPT (▲) along the post-inoculation (PI) period. Faecal score scale ranges from 0 (hard) to 4 (watery or with blood). [A,B] Different letters indicate statistical difference between days under Tukey adjustment (*P* <0.05).

Ileal histomorphometry

Table 4.3 shows the results of the experimental diets in ileal histomorphometry. High variability was observed in histological parameters with no significant differences between treatments in any of the parameters and days determined (*P* >0.22).

Table 4.3 Effect of experimental treatments on ileal histomorphometry on days 4 and 8 PI.

	Treatments				
	CTR	**BUT**	**HPT**	**RSE**	***P***
Villus height (µm)					
4 PI	163	220	200	82.5	0.391
8 PI	184	202	164	74.9	0.607
Crypt depth (µm)					
4 PI	238	227	223	37.5	0.740
8 PI	313	315	297	44.9	0.682
Villus:Crypt ratio					
4 PI	0.710	0.994	0.935	0.3749	0.303
8 PI	0.599	0.656	0.592	0.2897	0.889
IEL (cells/100 µm)					
4 PI	2.05	2.31	1.81	0.711	0.412
8 PI	3.17	2.73	3.27	0.672	0.222
GC (cells/100 µm)					
4 PI	1.19	1.74	1.47	0.892	0.469
8 PI	1.71	1.72	2.24	0.904	0.423
Mitoses[A] (cells/100 µm)					
4 PI	0.275	0.314	0.273	0.1755	0.892
8 PI	0.279	0.371	0.368	0.2129	0.625

[A] Mitoses in crypts. Legend: IEL, intraepithelial lymphocytes in villi; and GC, goblet cells in villi.

Immune response

Regarding serological concentrations of the pro-inflammatory cytokine TNF-α and the acute phase protein Pig-MAP, TNF-α was not modified by the experimental diets (*P* >0.20) with a mean value of 107 ± 54.0 pg/mL on day 4 PI, decreasing to 71.1 ± 19.02 pg/mL on day 8 PI. In the case of Pig-MAP, the values did not follow a normal distribution, and data were analysed as frequencies considering ranges of response reported by Piñeiro et al. (2009) including normal range (0.3 – 1 mg/mL), borderline (1 – 2 mg/mL) or high levels (>2 mg/mL). Only three animals showed concentrations above 2 mg/mL at day 4 PI. No significant differences were detected between diets (average of 1.20 ± 0.868 mg/mL and 0.73 ± 0.375 mg/mL on days 4 and 8 PI, respectively).

Heptanoic and butyric acid concentration along the gastrointestinal tract

Heptanoic acid was detected (*P* <0.001) in the stomach most of the animals receiving HPT diet (13/15) (Figure 4.2), with a mean concentration level of 1.02 ± 0.300 mmol/kg FM. It was also found in one animal receiving CTR (1/15) and another fed BUT (1/15) diet (0.040 and 1.500 mmol/kg FM, respectively). At duodenum, heptanoic acid was also detected (*P* <0.001) in most of the animals receiving HPT diet (11/13) with a mean concentration level of 0.05 ± 0.023 mmol/kg FM, and only in other two animals receiving CTR (1/14) or BUT treatment (1/15) (0.040 and 0.180 mmol/kg FM, respectively). In ileum, heptanoic acid could not be detected in any of the analysed animals, and in colon only in 8 animals (CTR 1/15, BUT 3/14, and HPT 4/14; *P* = 0.302) at very low levels (<0.1 mmol/kg FM).

Regarding the presence of butyric acid (Figure 4.2), in stomach it was detected (*P* <0.01) more frequently in animals fed with BUT (11/15) (5.09 ± 4.361 mmol/kg FM) than in those treated with CTR (6/15) or HPT (2/15) (3.98 ± 1.851 and 1.50 ± 1.117 mmol/kg FM, respectively). At the small intestine, butyric acid only could be detected in the ileum of one animal receiving BUT diet (1/7) at day 8 PI (0.420 mmol/kg FM). In colon, butyric acid was the second main SCFA, following acetic acid, representing between 19.5 and 45.7 % of total SCFA. No differences were seen, however, between treatments (*P* >0.05).

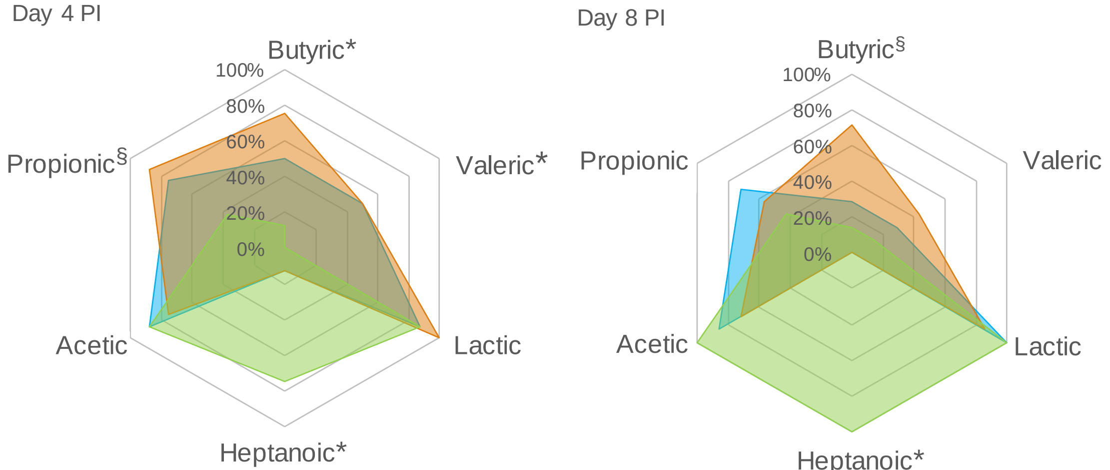


Figure 4.2 Percentage of piglets with detectable levels of each organic acid in stomach in the different experimental groups CTR (●), BUT (■), and HPT (▲). n = 8 animals and n = 7 animals for each diet at days 4 and 8 PI, respectively. § Statistical trend between treatments (0.10> *P* >0.05). * Statistical difference between treatments (*P* <0.05).

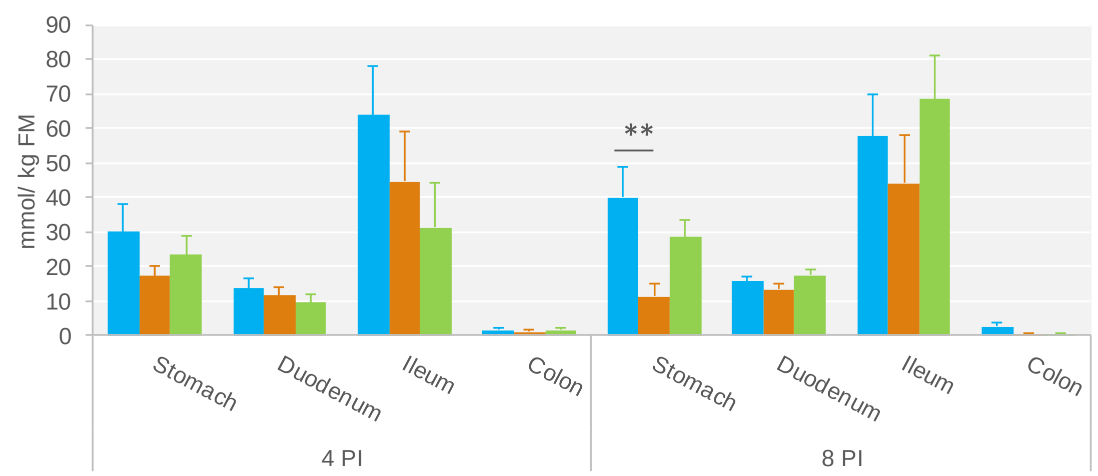
Fermentative activity along the gastrointestinal tract

The ileal pH was only slightly increased at day 8 PI with BUT (6.37, 6.56 and 6.37 for CTR BUT and HPT, respectively; *P* = 0.05). No changes were observed in colon. Ammonia concentration was not modified by any of the experimental diets in ileum, nor in colon (data not shown).

In Figure 4.3 are plotted the evolutions of the concentration of lactic acid and total SCFA along the gastrointestinal tract for the different experimental diets. At gastric level, an important fermentative activity was detected, being acetic and lactic acid the main fermentation products, ranging from unquantifiable levels in some animals up to 22.1 and 79.8 mmol/kg FM, respectively (Figure 4.3). Other acids, like propionic, butyric and valeric acid, were also found, although not in all the animals. Acetic acid was nearly the unique SCFA found in ileum ranging from 0 to 13.6 mmol/kg FM and was the main SCFA in colon representing 53.2 ± 5.36 % of total SCFA. Other SCFA in colon represented 31.7 ± 6.20, 11.6 ± 4.08, 2.36 ± 1.734 and 1.20 ± 1.029 mmol/kg FM for propionic, butyric, valeric and BCFA, respectively.

Regarding the effects of treatments on fermentative activity, sodium heptanoate (HPT) was able to modify fermentation at gastric level (Figure 4.2), tending to reduce the proportion of animals with detectable concentrations of propionic acid (6/15 vs. 11/15

and 11/15 for HPT, CTR and BUT, respectively; *P* = 0.10) and reducing the number of animals with detectable concentrations of valeric acid (1/15 vs. 6/15 and 7/15 for HPT, CTR and BUT, respectively; *P* = 0.02) and butyric acid (2/15 vs. 6/15 and 11/15 for HPT, CTR and BUT, respectively; *P* <0.01). The HPT treatment was also able to reduce the total amounts of SCFA in colon at day 4 PI (*P* = 0.01) but not at day 8 (*P* = 0.006) (Figure 4.3). No significant changes were detected in molar proportions of SCFA in this compartment.



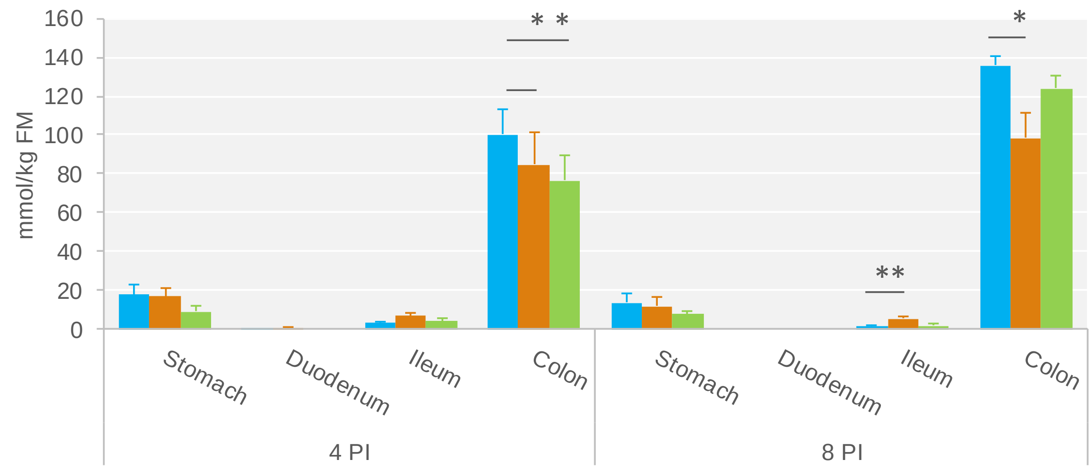


Figure 4.3 Lactic acid and total SCFA along the gastrointestinal tract in the different experimental groups CTR (●), BUT (■), and HPT (▲). § Statistical trend between treatment and CTR group under Dunnet adjustment (0.10> *P* >0.05). * Statistical difference between treatment and CTR group under Dunnet adjustment (*P* <0.05).

Regarding BUT diet, it was associated with a reduction in the concentration of lactic acid in stomach at day 8 PI (40.1, 11.4 and 28.4 mmol/kg FM for CTR, BUT and HPT, respectively; *P* <0.01) and an increase of acetic acid concentration at ileum (1.80, 5.20

and 1.89 mmol/kg FM for CTR, BUT and HPT, respectively; *P* = 0.05). At colon, like HPT, BUT tended to reduce total SCFA concentration at day 4 (*P* = 0.10) and reduced it at day 8 PI (*P* = 0.01), increasing the molar proportion of acetic (49.6, 54.4 and 48.4 % for CTR, BUT and HPT, respectively; *P* = 0.04).

Microbiological analysis

When animals arrived, faecal samples were analysed for enterobacteria and total coliforms with no differences registered between experimental groups (9.32 ± 0.812 and 9.04 ± 0.870 log CFU/g fresh matter [FM], respectively). After one week of adaptation, enterobacteria showed a numerical increase with BUT (7.03, 8.47 and 7.84 log CFU/g FM for CTR, BUT and HPT, respectively; *P* = 0.17), and total coliforms tended to be increased by HPT (6.34, 7.08 and 7.34 log CFU/g FM for CTR, BUT and HPT, respectively; *P* = 0.09).

Table 4.4 shows the plate counts of enterobacteria and total coliforms at ileal and colonic level on days 4 and 8 PI as well as the number of *E. coli* F4 measured by qPCR in ileal mucosa and colon digesta. In general, enterobacteria and coliforms counts decreased from day 4 to 8 PI. On day 4 PI, animals treated with both BUT and HPT exhibited higher numerical counts of enterobacteria in ileal mucosa (*P* = 0.041), although mean comparisons with CTR did not reach statistical significance. With BUT, *E. coli* was increased (*P* = 0.008), as well as with both additives *E. coli* from ileum digesta tended to be incremented on day 8 PI (*P* = 0.062). Regarding possible effects of the experimental treatments on *E. coli* F4 numbers, no significant differences were found in neither colon digesta nor mucosal scrapings.

Microbiota 16S rRNA gene analysis

Additionally, the high-throughput sequencing (HTS) of the 16S rRNA gene from the colonic DNA samples from day 4 PI was performed considering that the effects observed on fermentation and traditional microbiology were more evident on this sampling day (data shown above).

Table 4.4 Effect of the experimental treatments on enterobacteria and coliform plate counts and pathogenic *E. coli* F4 on days 4 and 8 PI.

		Treatments				
		CTR	**BUT**	**HPT**	**RSE**	***P***
Ileum mucosa	Enterobacteria (log CFU/g FM)					
	4 PI	4.97	6.15	6.13	0.978	0.041
	8 PI	5.35	5.88	5.29	0.822	0.312
	E. coli (log CFU/g FM)					
	4 PI	4.29[a]	5.87[b]	5.46[ab]	0.922	0.008
	8 PI	4.73	5.16	4.62	1.161	0.626
	E. coli F4[A] (log F4 copies/g FM)					
	4 PI	3.68	4.03	5.24	2.179	0.344
Ileum digesta	Enterobacteria (log CFU/g FM)					
	4 PI	7.98	9.11	8.96	1.722	0.381
	8 PI	6.36	7.14	6.84	0.723	0.120
	E. coli (log CFU/g FM)					
	4 PI	7.24	8.50	8.29	1.328	0.151
	8 PI	6.08	6.90	6.65	0.659	0.062
Colon digesta	Enterobacteria (log CFU/g FM)					
	4 PI	7.95	8.67	8.77	1.160	0.325
	8 PI	6.89	7.23	6.92	1.122	0.798
	E. coli (log CFU/g FM)					
	4 PI	7.44	7.78	8.13	1.102	0.474
	8 PI	6.24	6.78	6.61	1.066	0.595
	E. coli F4[A] (log F4 copies/g FM)					
	4 PI	7.57	8.19	8.23	1.886	0.742
	8 PI	5.05	5.34	5.20	0.833	0.789

[A] Pathogenic *E. coli* loads quantified as log F4 gene copies/g FM. *E. coli* F4 in ileal scrapings at day 8 PI was below the detection limit of the method (3.83-log F4 gene copy numbers/g). [a,b] Different letters indicate statistical difference between treatment and CTR group under Dunnet adjustment (P <0.05).

To avoid artefactual rare species, the analysis was based on the 836 OTU shared among all three experimental groups (OTU core of 89.4 %). The number of reads per sample was the same among the three treatments (P = 0.870) despite variability ranging from 29,516 to 295,283 no. reads/sample (mean of 84,765 no. reads/sample). All experimental groups also reached the plateau phase in rarefaction curves (data not shown) as well as did not show differences in Chao1 index (P = 0.708). The treatments did not reflect any changes in neither alpha diversity (Shannon index P = 0.530) nor beta diversity (P = 0.881) (data not shown).

In Figure 4.4 are presented the major represented phyla, families and genera detected among groups. Firmicutes (average 45.7 %) and Bacteroidetes (average 36.8 %) were the two major phyla followed by Proteobacteria (10.7 % average). At family level, 84.0 % of the OTU could be assigned to 45 families, and at genus level, 61.3 % of the OTU were assigned to 63 genera.

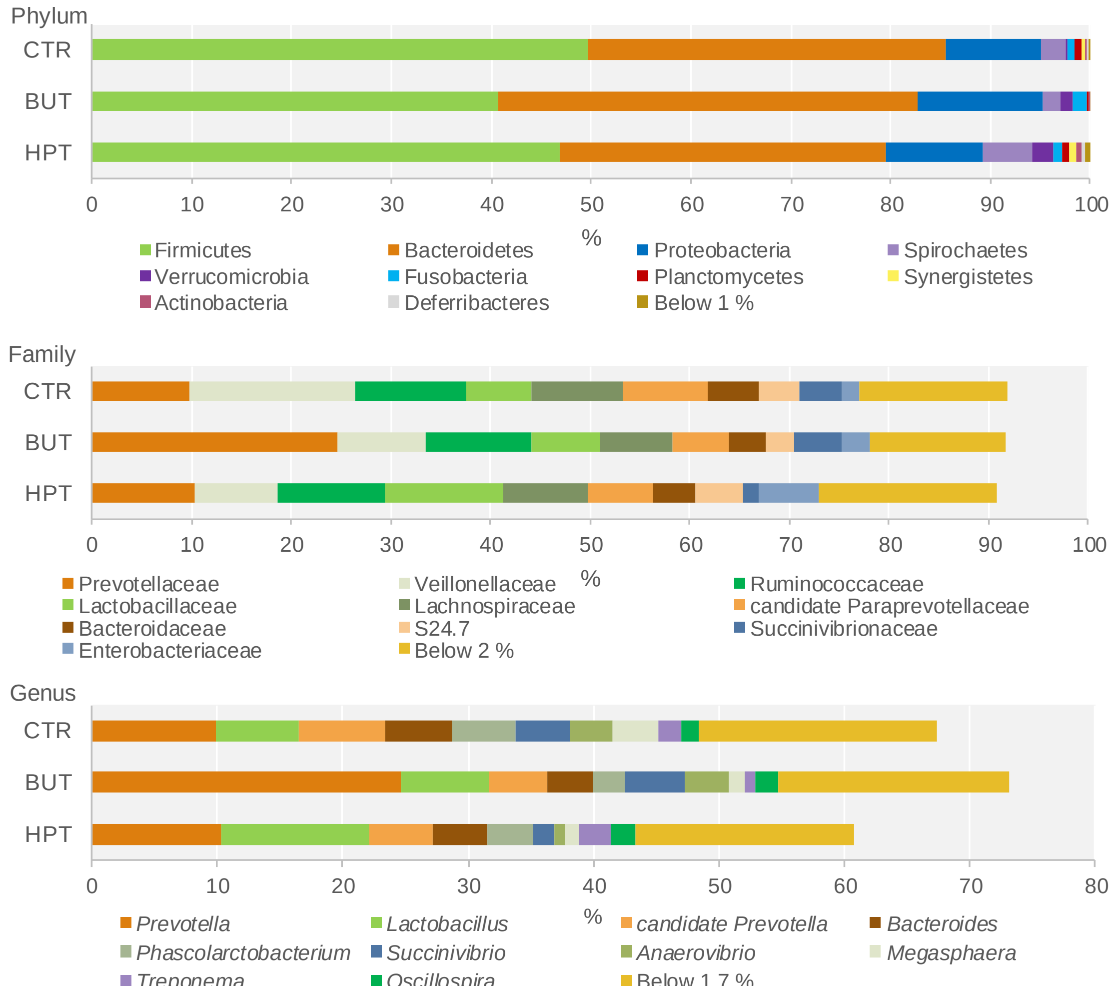


Figure 4.4 Mean relative abundance (%) of top 10: phyla; families; and genera, in the different experimental groups. The rest of the taxonomic groups are pooled together (those representing less than a mean of 1, 2 and 1.7 % of phyla, families and genera, respectively).

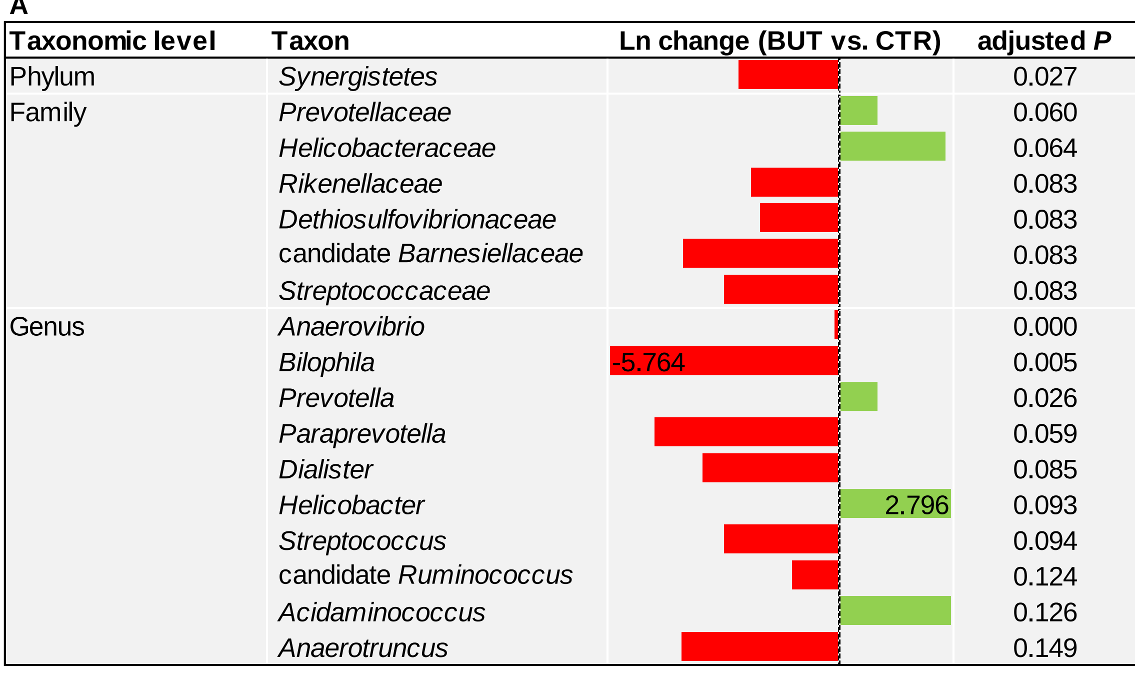
Some changes were detected on compositional taxa of the major groups related to the experimental diets. At phylum level, animals receiving the BUT diet, despite it was only a numerical trend, displayed a lower ratio of Firmicutes:Bacteroidetes (F/B) compared to the control group (0.978 in BUT vs. 1.531 in CTR; P = 0.111). These changes were the result of shifts observed with the BUT treatment at lower taxonomic levels, as an increase

in the proportion of Prevotellaceae (24.7 ± 7.77 % in BUT vs. 9.83 ± 5.32 % in CTR group; *P* = 0.060), a family belonging to Bacteroidetes phylum, and an increase in *Prevotella* genus (24.7 ± 7.77 % in BUT vs. 9.84 ± 5.31 % in CTR group; *P* = 0.026). Regarding HPT treatment compared to CTR, only a numerically higher F/B ratio was found (1.611 in HPT vs. 1.531 in CTR group; *P* = 0.842).

Most of the changes produced by the treatments were observed at minor taxa (<1.5 %), and therefore in Figure 4.5 are represented the specific taxa that were significantly modified by BUT or HPT treatment relative to CTR group. Those taxa detected in at least half of the animals per diet were those primarily evaluated.

Beyond the increase of Prevotellaceae and *Prevotella* by BUT treatment, Helicobacteraceae and *Helicobacter* were also promoted compared to CTR group (*P* <0.10). On the contrary, *Paraprevotella* genus within Prevotellaceae was decreased (*P* = 0.059), and the phylum Synergistetes and several families were reduced with BUT (*P* <0.10) including Dethiosulfovibrionaceae from this phylum, and Rikenellaceae, Barnesiellaceae, a candidate from the GreenGenes database, and Streptococcaceae and *Streptococcus* (*P* <0.10). Other genera were also reduced in animals treated with BUT including *Anaerovibrio* (*P* <0.001) and *Dialister* (*P* = 0.085), from Veillonellaceae family, and *Bilophila* (*P* = 0.005).

With HPT, the increments in *Collinsella* genus drove the increments of the corresponding family and phylum (Coriobacteriaceae and Actinobacteria, respectively). Opposite to this, Oxalobacteraceae and *Oxalobacter* as well as other genera such as *Roseburia* or *Parabacteroides* were decreased in the colon of HPT animals (*P* <0.10). Significantly differing were the genera *Paraprevotella* and *Lachnospira* (*P* <0.05), which were reduced with HPT.



A

Taxonomic level	Taxon	Ln change (BUT vs. CTR)	adjusted *P*
Phylum	*Synergistetes*		0.027
Family	*Prevotellaceae*		0.060
	Helicobacteraceae		0.064
	Rikenellaceae		0.083
	Dethiosulfovibrionaceae		0.083
	candidate *Barnesiellaceae*		0.083
	Streptococcaceae		0.083
Genus	*Anaerovibrio*		0.000
	Bilophila	-5.764	0.005
	Prevotella		0.026
	Paraprevotella		0.059
	Dialister		0.085
	Helicobacter	2.796	0.093
	Streptococcus		0.094
	candidate *Ruminococcus*		0.124
	Acidaminococcus		0.126
	Anaerotruncus		0.149

B

Taxonomic level	Taxon	Ln change (HPT vs. CTR)	adjusted *P*
Phylum	*Actinobacteria*		0.083
Family	*Coriobacteriaceae*		0.006
	Oxalobacteraceae		0.115
	Pasteurellaceae		0.146
Genus	*Paraprevotella*	-5.744	0.006
	Lachnospira		0.024
	Roseburia		0.053
	Oxalobacter		0.053
	Parabacteroides		0.062
	Collinsella		0.073
	Akkermansia	3.703	0.140

Figure 4.5 Differentially abundant taxa (ln change and FDR-adjusted *P* <0.15) between: BUT vs. CTR (A); and HPT vs. CTR (B). Positive values (■) and negative values (■) indicate greater and lower abundance, respectively, in treated animals (BUT or HPT). Taxa are sorted by level of significance (from higher to lower). Only extreme positive and negative ln changes are displayed as reference. Only taxa detected in at least half of the samples are represented. Taxa in brackets indicate a candidate classification.

4.4 Discussion

In this study, an experimental model of post-weaning colibacillosis was used to evaluate the efficacy of two protected forms of heptanoate and butyrate. As expected, the oral ETEC F4 inoculation prompted a reduction of feed intake, restored from day 5 PI onwards, and a mild course of diarrhoea accompanied with hypothermia of 48 h. Similar outcomes have been described in other studies of our group (Gustavo Hermes et al., 2013; Guerra-Ordaz et al., 2014; Barba-Vidal et al., 2017b) and by other authors with similar infective

doses (Risley et al., 1993; Spitzer et al., 2014; Rhouma et al., 2017). Serological levels of TNF-α were increased in the challenged animals at day 4 PI (range 101-111 pg/mL) within the values previously described (range between 79.2 – 110.2 pg/mL) (Hermes et al., 2013; Barba-Vidal et al., 2017b). Regarding the major acute-phase protein in pig, Pig-MAP, three animals presented acute values (>2 mg/mL) at day 4 PI but none at day 8 PI. Other authors have described quick and higher responses within the first 48 h after injury or stress (González-Ramón et al., 1995) and established a high baseline around the weaning period (Piñeiro et al., 2009). Our results suggest that our challenge was mild, and animals recovered quickly. Actually, at day 8 PI, most of the animals reduced TNF-α levels and had normal Pig-MAP values.

When considering effects of experimental diets on performance, no differences were observed between treatments on ADFI or ADG achieving similar final body weights. Reference should be made to the low ADG values observed during pre-inoculation period with high RSE values that would reflect the detrimental effect of post-weaning stressors in some particular pens. Two pens from CTR and BUT and three from HPT presented abnormal low performances reflecting that animals adapted differently to facilities and to weaning. This high variability would have required a higher number of replicates to evaluate effects on performance, and therefore the absence of significant effects on growth rate should be regarded with caution. Actually, other studies evaluating the effect of butyric acid or its sodic salt on pig performance have demonstrated positive effects on performance (Manzanilla et al., 2006; Lu et al., 2008), although the results were not always improved (Biagi et al., 2007; Weber & Kerr, 2008; Mallo et al., 2012). Differences in the doses, basal diets and environmental conditions could explain this variability. Regarding sodium heptanoate, to our knowledge there is only one *in vivo* study analysing performance effects of heptanoic acid in combination with nonanoic acid (C9) in piglets (De Smet et al., 2016), and the authors were not able to find positive effects.

Regarding the release of supplemented acids along the GIT, it was possible to detect increases in the concentration of butyric acid in stomach with the BUT diet and also increases in heptanoic acid in stomach and duodenum with the HPT diet. In the line of our results, other authors were only able to find increases in the concentration of the supplemented protected acids in the upper gastrointestinal tract but not in the hindgut

(Dierick et al., 2003; Manzanilla et al., 2006; Mazzoni et al., 2008; Zentek et al., 2013; De Smet et al., 2016). It should be borne in mind that SCFA and particularly butyric acid are absorbed quickly by the intestinal mucosa, particularly at the hindgut (Argenzio & Southworth, 1975; Schmitt et al., 1976; Bugaut, 1987). Although a slow release of the acid is expected with the protected forms, the quick absorption of the gradually released acids might have drained them from the gut lumen making it impossible to detect differences in the luminal concentration. However, despite the inability to detect differences, most of published works evidence ability of the in-feed additives to modify different variables along the whole GIT.

In this regard, our results consistently support the ability of the supplementation with BUT or HPT to modify the microbial activity all along the whole gut. Regarding effects on the stomach, the inclusion of butyrate and heptanoate salts had a significant impact on stomach fermentation with changes in the SCFA profile. In the case of HPT, a lower number of animals had quantifiable levels of propionate and valerate, which could be due to a selective inhibition of certain bacterial populations specialised in propionic or valeric production (Ríos-Covián et al., 2016). Similarly, other authors (Dierick et al., 2004; Zentek et al., 2012) have also observed decreases in propionate and valerate in the upper small intestine with the most common MCFA tested (caprylic and capric acids). Regarding the effects of BUT, it is worth mentioning the significant reduction observed in lactic acid concentration in the stomach at day 8 PI (11.4 vs. 40.1 mM) (P <0.01), that was also numerically maintained at ileum with a significant increase in luminal pH. These results would suggest a decrease of the lactobacilli population as one of the main lactic acid bacteria (LAB) in the stomach (Mann et al., 2014; Holman et al., 2017). Another likely scenario would be the enrichment of lactate-using butyrate-fermenter bacteria (Metzler-Zebeli et al., 2011; den Besten et al., 2013) lowering lactic acid concentration in stomach and increasing the formation of butyrate, which is quickly absorbed in this organ (Castillo et al., 2006; Manzanilla et al., 2006; Mazzoni et al., 2008). However, as we did not analyse microbial changes in the stomach we cannot confirm any of these hypotheses.

Although specific microbial changes produced in the stomach were not identified, both additives had a significant impact on gastric fermentation. Here it is interesting to point out the relevant gastric fermentative activity found in some animals measured as total

SCFA and lactic acid with values that reached up to 36.3 mM for SCFA and 79.8 mM for lactic acid. Traditionally, it has been considered that the fermentative activity in the stomach of monogastric animals, like pigs, was scarce, particularly in the young ones (Barro et al., 1977; Gálfi & Bokori, 1990). However, our results confirm the relevance of the gastric fermentation in post-weaning piglets. It could be reasonable to think that in the young pig, due to the low acidifying activity of the stomach (Barrow et al., 1977), a quick growth of certain microbial groups could be produced at particular times post-feeding. This stomach fermenting microorganisms could later colonise forward parts of the gut and have a profound impact in the whole GIT ecosystem. In this regard, the stomach microbiota and its fermentative activity have recently attracted the interest of the scientific community (Metzler-Zebeli et al., 2011; Metzler-Zebeli et al., 2013; Mann et al., 2014; Xu et al., 2016). A high microbial biodiversity in stomach has been related to a better health status, being considered as a potential biomarker of wellbeing (Ríos-Covián et al., 2016).

Downward into the ileum compartment tested additives also promoted significant changes in microbial activity. Total SCFA were almost tripled by the addition of BUT mostly due to an increase in acetic acid. A selective promotion of acetogenic bacteria and the consequent conversion of supplemented butyrate to acetic acid seem plausible (Bergman, 1990). Increases of acetic acid found in ileum and colon have also been described by other authors with the supplementation of butyrate (Gálfi & Bokori, 1990; Manzanilla et al., 2006). Although HTP did not modify SCFA concentration at ileal level, similarly to BUT both in-feed additives increased the number of enterobacteria and total coliforms in ileum content and mucosa. This was an unexpected result as both microbial groups were selected as markers of potential pathogens as ETEC F4. Several studies supplementing sodium butyrate had reported a reduction of pathogens such as *Clostridium* and *E. coli* in small and large bowel in pigs (Lu et al., 2008) and even sodium heptanoate *in vitro* against *Campylobacter jejuni* (Grilli et al., 2013). However, not necessarily an increment in coliforms and enterobacteria means an increase in the pathogen challenge. Actually, *E. coli* is a natural commensal in the gastrointestinal tract of the pig (Heo et al., 2013) that has also been described as one of the first colonisers of the gut that generates the proper environment for future colonising bacteria (Petri et al.,

2010). Actually, when *E. coli* F4 by qPCR was quantified specifically no increase was detected in the colonisation of the gut with the experimental additives and neither evidence of tissular damage. No changes were detected in the histomorphological parameters and neither in TNF-α or Pig-MAP. These results suggest that the increases registered in coliforms and enterobacteria not necessarily meant an increase in the pathogen gut colonisation. Moreover, one could even hypothesise that in some cases an increase in certain coliforms could be beneficial against colonisation of the gut by pathogenic strains. In this regard, it has been shown how some *E. coli* strains can limit the infective ability of the pathogenic ones, phenomenon known as colonisation resistance (Leatham et al., 2009). In the case of BUT, the increase in ileal coliforms and enterobacteria could also be related to the decrease in lactic acid registered in stomach and ileum, which suggests a reduction of gastric lactobacilli that could have favoured the colonisation of the foregut by enterobacteria and coliforms.

The experimental in-feed additives also promoted significant changes at colonic level. Remarkable in this compartment was the decrease observed with both additives in the concentration of total SCFA. This reduction was more consistent with BUT, being detected at both sampling days (4 and 8 PI). Reductions in the concentration of SCFA could be regarded as the result of a reduction in the amount of undigested material arriving to the hindgut. Some organic acids have shown to delay the gastric emptying (Hunt & Knox, 1969; Manzanilla et al., 2004) favouring the pre-digestion of diets at the end of the small intestine and thus reducing the available substrate arriving to the hindgut to be fermented. Other works with supplemented sodium salts have not described similar decreases of SCFA concentration (Canibe et al., 2003; Biagi et al., 2007); however, this could be due to differences in manufacturing technology, doses, way of administration, development and maturity of the GIT and microbial ecosystem, and in our case the ETEC F4 challenge.

Changes in colonic SCFA registered with both additives could also reflect changes in colonic microbiota structure. Actually, previous works of our group (Castillo et al., 2006) also reported significant changes in the composition, ecological structure and metabolic activity of the hindgut microbiota when supplementing another form of sodium butyrate to post-weaning piglets. In that work, it was suggested that an increase of digestion and

absorption of nutrients in the upper GIT could potentially have modified the fermentable material reaching the large intestine and therefore the colonic ecosystem. To our knowledge, in the literature there are only two works evaluating the effects of sodium salts, particularly sodium butyrate and in combination with propionate and MCFA, on the gut microbiota of the pig through the HTS of the 16S rRNA gene (Xu et al., 2016, Soler et al., 2018). In those works, it was observed that most of the changes were taking place in the stomach but also in distal parts (ileum or colon and faeces), possibly derived from the idea previously mentioned of the consecutive colonisation of the gut of the young piglets.

In the present work, HTS results showed that, in general terms, the diversity and community structure of colonic microbiota was according to the predominant taxa described previously for healthy and adult pigs (Holman et al., 2017). This general overview did not show relevant changes that could suggest acute dysbiosis and point out the resilience of main microbial groups even in stressor situations as weaning (Han et al., 2018) or the oral challenge in this study. Regarding the impact of the experimental additives on colonic microbiota, even though no significant changes in the ecosystem structure or diversity were found, important shifts were observed in specific populations. The most prominent was the relative increase of Prevotellaceae and *Prevotella* in the colon digesta of animals treated with BUT resulting in a lower F/B ratio. *Prevotella* is the major genus found among all the GIT sites despite of age (Xiao et al. 2016; Han et al., 2018) and represents the shift towards the microbiota developed after weaning (Mach et al., 2015). *Prevotella* is shown to digest complex carbohydrates to produce acetate (Holman et al., 2017) and has been associated to one of the two enterotypes described in pigs that correlates positively with the immune response and performance (Mach et al., 2015). In our work, acetate was increased in both ileum and colon also with BUT, although we were not able to demonstrate effects on performance or immunity. *Prevotella* increase promoted by BUT was concomitant with the decrease of other bacterial populations such as Rikenellaceae (Su et al., 2014), *Streptococcus* (Koh et al., 2016) or *Paraprevotella* that occupy the same functional niche as *Prevotella*. With HPT, the most consistent effect was the increase observed in Actinobacteria phylum, and the corresponding Coriobacteriaceae family driven by *Collinsella* genus within. This genus has been related to human metabolic disorders (Gomez-Arango et al., 2018) but detected in

very low amounts in the gut of the pig (<0.1 %) (Holman et al., 2017). Nonetheless, Actinobacteria are typical from the upper gut and generally produce acetate but not propionate nor butyrate (Binda et al., 2018). On the other hand, and in the same vein as BUT, HPT decreased *Paraprevotella* as well as Lachnospiraceae genera (*Lachnospira* and *Roseburia*), belonging to Clostridiales. This group is comprised of butyrate-producers that ferment acetate (Vital et al., 2014). In global terms, it could be hypothesised a carry-over effect from HPT reflecting those changes observed in gastric fermentation (propionic and butyric depression) towards colon, however, we were not able to detect significant changes in the molar proportion of these colonic SCFA, neither acetic acid, with HPT diet. Moreover, these changes were not consistent across the two days as occurred with BUT and must be read with caution considering that these modified groups only represent a minimal proportion of the total population (mean of <0.4 %) compared to *Prevotella* (mean of 15 %).

4.5 Summary

The protected forms of sodium butyrate and heptanoate tested in this work were demonstrated to be able to modulate the fermentative activity along the entire gastrointestinal tract in weaned piglets under an oral ETEC F4 challenge. Particularly, BUT showed significant decreases of lactic acid in stomach and ileum promoting a more acetic fermentation at the end of the small gut accompanied with a specific promotion of *Prevotella* in colon. HPT also showed to modify gastric fermentation and promote typical gastric populations such as Actinobacteria in colon. Both additives were shown to increase the coliform populations in ileum, possibly limited to a non-harming commensal presence considering that no detrimental effects were found in the ileal histomorphometry and neither in the numbers of *E. coli* F4 determined by qPCR. Both salts showed significant decreases in the SCFA concentration at colon that could be related to changes in the foregut like hypothesised improvements in nutrient digestion that may have resulted in a decrease in fermentable substrate arriving to the hindgut. Although under our experimental conditions we were not able to demonstrate eventual specific benefits of the tested in-feed forms of sodium butyrate or heptanoate in front of

the oral ETEC F4 challenge, results suggest underlying adaptive drifts of the microbial and fermentative patterns towards weaning. More studies would be needed to better understand the mechanisms behind the effects observed.

Chapter 5 Efficacy of salts of medium-chain fatty acids distilled from coconut oil against two enteric pathogens in weaned piglets

5.1 Introduction

In modern pig production, early weaning around four weeks of life is associated with an abrupt dietary change and an immature organ function with a reduction of the growth and thus, piglets around the post-weaning period are highly susceptible to enteric bacterial infections caused by opportunistic pathogens. The use of antibiotics and other medication to face these problems has been common in the intensive systems, however, it has contributed to the development of antibiotic-resistant strains. Indeed, the last report from EFSA of the situation in 2015 documented about the increase of multi-drug resistant (MDR) strains of *Escherichia coli* and *Salmonella* Typhimurium (EFSA & ECDC, 2017). It is urgent therefore to look for alternatives that will diminish the diarrhoea outbreaks and will help to reduce the use of therapeutic and prophylactic antibiotics.

Feeding strategies are one of the most commonly used management factors for the improvement of the gut health and function of newly pigs. A wide range of bioactive ingredients have been proposed as tools to control digestive pathogens helping to reduce the incidence and severity of digestive problems associated with the weaning (Heo et al., 2013). Between them, medium-chain fatty acids (MCFA), considered as those fatty acids from 6- to 12-carbon atoms chain-length, have been proposed as a potential alternative to antibiotics based on their long known antibacterial activity (Skrivanová et al., 2006). In contrast with antimicrobial agents, MCFA have not shown evidence of resistance acquirement (Petschow et al., 1996; Manohar et al., 2013). Besides this, MCFA are an immediate source of energy due to the rapid passive absorption and digestion with a particular interest in nutrition of young animals (Li et al., 2015; Schönfeld & Wojtczak, 2016). They occur naturally as medium-chain triglycerides (MCT) in milk fat as well as in other vegetable sources such coconut or palm oils and *Cuphea* seed oil (DebMandal & Mandal, 2011; Baltić et al., 2017). The most abundant MCFA in coconut is lauric acid (C12), representing up to 45 % of the coconut fat content followed by capric (C10) and caprylic (C8) acids (Zentek et al., 2011). The C12 acid has been proven as the greatest antimicrobial component among all MCFA (Petschow et al., 1996; Vande Maele et al., 2016).

Several published works can be found substituting fat sources with purified MCFA or alternative natural sources rich on MCFA showing improvements in performance of piglets around weaning (Hernández & Pluske, 2008; Weng et al., 2017). Studies with healthy pigs (Dierick et al., 2002a; Hanczakowska et al., 2011; Kuang et al., 2015) have demonstrated positive effects on growth performance, digestibility, or intestinal microbiota. The antibacterial effect has also been widely demonstrated *in vitro* (Dierick et al., 2002b; Skrivanová et al., 2006) and particularly against *Salmonella* or enterotoxigenic *E. coli* (ETEC), among other pathogens (Messens et al., 2010; Martínez-Vallespín et al., 2016). However, up to our knowledge, most of the *in vivo* studies testing MCFA effect against pathogens were performed in rodents (Kono et al., 2004; Manohar et al., 2013) and chickens (Van Immerseel et al., 2004). Only one work was found that tested the potential of MCFA to control *Salmonella* in pigs (Rasschaert et al., 2016). Within this context, the objective of this work was to evaluate the potential of a combination of sodium salts of medium-chain fatty acids distilled from coconut oil to enhance gut health of weaned piglets in front of diarrhoeic enteric diseases caused by *Salmonella* or ETEC F4 in controlled clinical assays.

5.2 Material and methods

Two different experiments were performed to evaluate the effect of the coconut distillates against an oral challenge with *Salmonella* Typhimurium (Trial 1) or ETEC F4 (Trial 2). Both trials were performed at the Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (UAB) and received prior approval from the Animal and Human Experimental Ethical Committee of this Institution (permit no. CEAAH 2933). The treatment, management, housing, husbandry and slaughtering conditions conformed to European Union Guidelines (Directive 2010/63/EU).

Animals, housing and experimental design

The two trials were conducted following Biosafety Level 2 requirements with appropriate training of the involved personnel. For each trial, 48 male piglets were used from high

sanitary status farms. For the first trial, [Landrace x Large White] x Piétrain piglets came from mothers with negative results on *Salmonella* serology weaned at 28 days of age and 8.1 ± 1.16 kg of body weight (BW). For the second trial, [Landrace x Large White] x Piétrain piglets came from non-vaccinated mothers against *E. coli* weaned at 21 days of age and 5.6 ± 0.95 kg BW.

Piglets were transported to the UAB facilities and placed in 16 pens (three animals per pen). In each pen, animals were distributed by weight (a low-, an intermediate- and a high-weighted animal) to obtain a final homogenous weight among pens. The experimental treatments included a control treatment (CTR) consisting of a plain diet without additives and the same diet including a commercial blend of salts of MCFA distilled from coconut oil (Dicosan) at 3 kg/t (DIC). Experimental treatments were evenly distributed among the pens.

Each pen (3 m^2) had a feeder and a water nipple to provide food and water for *ad libitum* consumption. The weaning rooms were equipped with automatic heating and forced ventilation. The experiments were conducted during the autumn-winter seasons (September – October and February – March for Trial 1 and 2, respectively) under a mean room temperature of 30.8 ± 5.28 ºC. Both trials were maintained under a 13 h light/11 h dark lighting regimen.

Experimental products and diets

The evaluated feed-additive is commercially available (Dicosan) and was supplied by Norel S.A (Madrid, Spain). It consists of a mixture of sodium salts of fatty acid distillates from coconut (67 % crude fat and minimum content of lauric acid of 32 %) obtained from distillates of coconut oil.

Basal diet (Table 5.1) was formulated to satisfy the nutrient requirement standards for pigs (NRC, 2012). For each trial, the diet was manufactured in the same batch and the additive was mixed with the corresponding amount of feed to obtain the DIC diet. Dicosan was included following the manufacturer’s recommended dosage (3 kg/t) equivalent to 1.11 kg/t of lauric acid.

Table 5.1 Ingredient composition (g/kg) and chemical analysis (%) of the basal diet as-fed basis.

Ingredients			
Maize	205.0	Mono-calcium phosphate	6.5
Wheat	180.0	Calcium carbonate ($CaCO_3$)	3.8
Barley 2 row	170.0	L-Lysine HCL	4.5
Extruded soybean	150.0	Vitamin-Mineral Premix[A]	4.0
Sweet whey-powder (cattle)	112.0	Sodium chloride (marine salt)	2.4
Fishmeal LT	60.0	DL-Methionine 99	2.6
Soybean meal 47	70.0	L-Threonine	2.3
Whey powder 50 % fat	25.0	L-Tryptophan	0.7
Calculated nutrients			
Metabolisable energy (kcal/g)	3315.4	Digestible lysine	13.9
Crude protein	211.1	Digestible methionine plus cysteine	8.2
Calcium	6.5	Digestible threonine	9.0
Digestible phosphate	4.1	Digestible tryptophan	2.8
Analysed chemical composition (%)	*Salmonella* trial	ETEC F4 trial	
Dry matter	902.1	915.0	
Ash	54.2	48.2	
Crude fat	59.4	66.5	
Crude protein	198.1	194.7	
Neutral detergent fibre	84.3	81.7	
Acid detergent fibre	31.1	31.5	

[A] Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D3, 39.9 mg vitamin E, 3 mg vitamin K3, 2 mg vitamin B1, 2.3 mg vitamin B2, 3 mg vitamin B6, 0.025 mg vitamin B12, 20 mg calcium pantothenate, 60 mg nicotinic acid, 0.1 mg biotin, 0.5 mg folic acid, 150 mg Fe, 156 mg Cu, 0.5 mg Co, 120 mg Zn, 49.8 mg Mn, 2 mg I, 0.3 mg Se.

Bacterial strains

For the first trial, the *Salmonella* strain used for the oral challenge was a monophasic variant of *Salmonella* Typhimurium (4,5,12:i:-) with a ACSSuT-Ge resistance profile and phage type U302, isolated from a salmonellosis outbreak in fattening pigs in Spain (mainly enteric with sporadic septicaemia) and provided by the Infectious Diseases Laboratory of UAB (ref. 301/99). The oral inoculum was prepared by overnight incubation at 37 ºC and 250 rpm in buffered peptone water (Oxoid) and diluted (1:20) with sterile phosphate buffered saline (PBS) (Sigma-Aldrich). The final inoculum concentration was 5.8 x 10^7 CFU/mL. To confirm doses serial dilutions of the inoculum were cultured in tryptic soy agar (TSA) (BD Difco) by overnight incubation at 37 ºC.

For the second trial, the enterotoxigenic *Escherichia coli* (ETEC) F4 strain used (positive for virulence factors F4ab, F4ac, LT, STb and EAST1 and negative for F6, F18, F41, STa, VT1, VT2 and EAE) was isolated from 14-week old pigs and provided by Diseases

Laboratory of UAB (ref. 30/14-3). The oral inoculum was prepared by overnight incubation at 37 ºC and 250 rpm in brain heart infusion (BHI) (Oxoid). The final inoculum was 2.5 x 10^8 CFU/mL. To confirm doses serial dilutions of the inoculum were cultured in Luria agar (LA) by overnight incubation at 37 ºC.

Experimental procedure

Animals received the experimental diets *ad libitum* over 14 days in the *Salmonella* trial and 15 days in the ETEC F4 trial. After 6 and 7 days of adaptation in the first and second trials, respectively, animals were orally challenged with the pathogen. One pig per pen was euthanised on days 4 and 8 post-inoculation (PI).

Faecal samples for microbiological analysis were aseptically collected from the heaviest piglet of each pen (N = 16) at arrival and after the adaptation period (day 0 PI) after spontaneous defecation or by digital stimulation. After the adaptation period, the pathogenic bacteria inocula were administered by oral gavage to all animals as a single dose of *Salmonella* Typhimurium (1.2 x 10^8 CFU) or ETEC F4 (1.48 x 10^9 CFU) on days 7 and 8, respectively. In order to ensure that the stomach was full at the time of inoculation and facilitate bacterial colonisation, feed withdrawal was done at 21:00 of the previous day and provided back 30 minutes before inoculation the following morning.

Individual BW and pen feed consumption were registered during the adaptation period. Body weight was further recorded on days 0, 4 and 8 PI in both trials while feed consumption on days 0, 2, 4 and 8 PI for *Salmonella* trial and 0, 1, 2, 3, 4, 5, 7 and 8 PI in the case of ETEC F4 trial. Average daily gain (ADG) and average daily feed intake (ADFI) were calculated per pen considered the experimental unit.

After the oral challenge, animals were checked daily for clinical signs to evaluate their status (i.e. dehydration, apathy and faecal score) always by the same person. Mortality was also registered, and no antibiotic treatment was administered to any of the animals in any of the experiments. Faecal score was measured using a scale: 1 = solid and cloddy, 2 = soft with shape, 3 = very soft or viscous liquid and 4 = watery or with blood. Faecal score was registered individually on days 0, 1, 2, 3 and 7 PI in *Salmonella* trial and on days

0, 1, 2, 3, 5 and 7 PI in ETEC F4 trial. Rectal temperature was assessed with a digital thermometer (Accu-Vet, Import Vet S.A) on days 0 and 2 PI. Within *Salmonella* trial, additional faecal samples for microbiological analysis were collected from the same animals on days 1, 3 and 7 PI.

At days 4 and 8 PI, one pig per pen was euthanised sequentially during the morning (between 8:00 and 14:00) for sampling. On day 4 PI, the animal of each pen with mid-weight at the beginning of the experiment, and on day 8 PI that with the highest weight were selected. A 10-mL blood sample was obtained by venepuncture of the cranial vena cava using 10-mL tubes without anticoagulant (Aquisel) and immediately animals received an intravenous injection of sodium pentobarbital (200 mg/kg BW) (Euthasol, Esteve). Once dead, animals were bled, the abdomen was immediately opened, and the intestinal tract excised.

Digesta content from ileum and proximal colon (5 cm from caeco-colic junction) was collected, homogenised and pH immediately determined with a pH-meter (Crison 52-32 electrode, Net Interlab), calibrated on each day of use. Different aliquots were collected, for different determinations. Samples of approximately 5 g were kept immediately on dry ice until being stored at -20 ºC for further analysis of short-chain fatty acids (SCFA) and lactic acid as well as similar aliquots stored at -80 ºC for high-throughput sequencing (HTS) of colonic microbiota. Another set of samples were preserved in a H_2SO_4 solution (3 mL of digesta plus 3 mL of 0.2 M H_2SO_4) and stored at -20 ºC until ammonia (NH_3) determination.

In the ETEC F4 trial, additional aliquots (approximately 2 g) from ileum and colon digesta were also kept on ice for microbiological analysis, being processed in less than 4 h, and other similar aliquots stored at -80 ºC for *E. coli* F4 quantification by real-time (quantitative PCR [qPCR]) and also for HTS of colonic microbiota. In parallel, to analyse bacteria attached to the ileal mucus and epithelium, 15-cm long section of ileum were excised, washed thoroughly three times with sterile PBS, opened longitudinally and scraped with a microscope glass slide to obtain the samples. One aliquot was kept on ice for microbial analysis. In the case of *Salmonella* trial, additional caecum digesta samples were collected and kept on ice being processed in less than 4 h for *Salmonella* counts.

For the histological study, 3-cm long sections from the distal ileum were removed, opened longitudinally, washed thoroughly with sterile PBS and fixed by immersion in a formaldehyde solution 3.7-4 % (PanReac).

Blood samples were centrifuged (1500 x *g* for 15 min) and serum obtained was divided in different aliquots and stored at -20 ºC.

Analytical procedures

Chemical analyses of the diets including dry matter (DM), ash, crude protein and diethyl ether extract were performed according to the Association of Official Agricultural Chemists standard procedures (AOAC, 2007). Neutral detergent fibre and acid detergent fibre were determined according to the method of Van Soest et al. (1991).

For microbial counts of *Salmonella* spp. (Trial 1), caecum contents and faeces were suspended in buffered peptone water (BPW) (1:10). Quantitative determination consisted in 10-fold serial dilutions in PBS seeded in Xylose-Lactose-Tergitol-4 (XLT4) agar (Merck) and the count of H_2S positive colonies and compatible morphology with *Salmonella* spp. after 24 h incubation at 37 ºC. With this scheme, animals were given a count level as follows: negative, for animals with no *Salmonella* growth at 10^2 dilution ($<10^3$ CFU/g fresh matter [FM]); low, for animals with counts from 10^3 to 10^4 CFU/g FM; medium, for animals with counts from 10^5 to 10^6 CFU/g FM; and high, for counts from 10^7 to 10^8 CFU/g FM.

For microbial counts of enterobacteria and total coliforms (Trial 2), ileum and colon contents and faeces were suspended in PBS (1:10) and serially diluted in Lactated Ringer's solution (Sigma-Aldrich) and seeded in MacConkey agar and chromogenic agar (Oxoid), respectively. Counts were read after 24 h incubation at 37 ºC.

In addition, for microbial molecular analysis DNA from samples (approximately 250 mg) was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen), and subsequently its concentration and purity checked using NanoDrop 1000 Spectrophotometer (Thermo Fisher). The protocol was followed applying all

recommended optimisation steps in order to improve bacterial cell rupture and purity. The DNA was finally eluted in 200 mL of Qiagen buffer AE and stored at -80 ºC until use.

E. coli F4 was quantified in colonic digesta and ileal scrapings by qPCR using SYBR green dye. A qPCR targeting the gene coding the F4 fimbria of ETEC F4 was performed by modifying the procedure described by Gustavo Hermes et al. (2013). Changes consisted in using SYBR Green PCR Master Mix PCR (Applied Biosystems) and reducing the total volume of reaction to 20 µL, which included 10 µL of 2X SYBR Green PCR Master Mix PCR buffer, 0.88 µL of each primer (12.5 µM) and 5 µL of DNA sample.

In the case for HTS of colonic microbiota in both trials, V3-V4 region of 16S rRNA was targeted using the kit MiSeq® Reagent Kit v2 (500 cycle) (MiSeq from Illumina). Primers used in the construction of libraries with amplicons of putative 460 bp were the following:

F-5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

R-5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Sequence reads of 16S rRNA gene generated from MiSeq Illumina® system were processed on QIIME v.1.9.1 pipeline (Caporaso et al., 2010) with default settings. The quality filter of already demultiplexed sequences was performed at a maximum unacceptable Phred quality score of Q20. Resulting reads were clustered to operational taxonomic unit (OTUs) using uclust with 97 % sequence similarity and subsampling pick open reference method (Rideout et al., 2014) at 10 % of sequences subsampled. Representative sequences were assigned to taxonomy against bacterial 16S GreenGenes v.13.8 reference database (DeSantis et al., 2006) at a 90 % confidence threshold and sequence alignment and phylogenetic tree building were obtained through uclust and FastTree. Thereafter, chimeric sequences were removed with ChimeraSlayer (Haas et al., 2011) with default settings and further quality filtering consisted in removing singletons and OTUs with relative abundance across all samples below 0.005 % as recommended by Bokulich et al. (2013).

Ammonia (NH_3) concentration was determined with the aid of a gas-sensitive electrode (Hach Co.) combined with a digital voltmeter (Crison GLP 22, Crison Instruments S.A) modified from Barba-Vidal et al. (2017a). Three grams of digesta preserved in H_2SO_4 (1:2) were centrifuged at 1372 x *g* for 10 minutes. Supernatant was obtained and finally

neutralised with 1 mL of 10 M NaOH to reach pH 11 while stirring and measuring ammonia released as different voltage in mV.

SCFA and lactic acid were determined based on the method of Richardson et al. (1989) modified by Jensen et al. (1995) performed by gas chromatography after submitting the samples to an acid–base treatment followed by an diethyl ether extraction and derivatisation with *N*-(tertbutyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MBTSTFA) plus 1 % *tert*-butyldimethylchlorosilane (TBDMCS) agent (Sigma-Aldrich).

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned at 4-µm thickness and stained with haematoxylin and eosin. Morphological measurements of ten different villus-crypt pairs were performed with a light microscope (Leica DM5000B, Jenoptik) fitted to a CapturePro software (ProgRes®, Jenoptik) using the technique described in Nofrarías et al. (2006).

Serum concentrations of tumour-necrosis factor-α (TNF-α) were determined by Quantikine Porcine TNF-α kits (R&D Systems); and pig-major acute-phase protein (Pig-MAP) concentration was determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A). In *Salmonella* trial, serological antibodies of *Salmonella* were tested by ELISA *Salmonella* Herdchek (Idexx).

Statistical analysis

The effect of experimental treatments on performance and slaughter measurements was performed in free software R v3.4 using the stats package (R Core Team, 2013) lm() function for one-way ANOVA with diet as fixed effect. For microbial analysis, in Trial 2, enterobacteria and coliform counts were log transformed and followed the same one-way ANOVA analysis. In the case of Trial 1, *Salmonella* spp. count levels were analysed with stats package likelihood.test() function for frequency analysis and the number of positive animals to *Salmonella* followed the stats package glm() function under a binomial distribution. Pig-MAP concentration levels were also analysed with the same frequency analysis considering normal range (0.3 – 1 mg/mL), borderline (1 – 2 mg/mL) and high levels (>2 mg/mL) (Piñeiro et al., 2009), and the number of animals with detectable copies

of the pathogen F4 gene or quantifiable levels of fermentation products were analysed under the binomial distribution.

Average daily feed intake, rectal temperature and daily faecal consistency were performed using the lme4 package (Douglas et al., 2015) lmer() function for adjusted linear mixed model with a treatment-by-time interaction term.

For all analysed data, the pen was used as the experimental unit. The alpha level for the determination of significance for all the analysis was 0.05. The statistical trend was also considered for *P* values >0.05 and <0.10. Data are presented as means and residual standard error (RSE).

Biostatistics of quality-filtered sequences were performed in open source software R v.3.4.3. Firstly, OTU table was imported to R with phyloseq package (McMurdie & Holmes, 2013). Only OTUs shared among experimental groups were included and unique OTUs excluded. Diversity and ordination analysis (non-multidimensional scaling, NMDS) were analysed at OTU level using vegan package (Oksanen, 2017). Richness and alpha diversity were calculated with raw counts based on Chao1 estimator (Chao, 1984) to estimate the number of taxa in the community and the Shannon index (Shannon & Weaver, 1963) that considers the richness as well as the evenness. For beta diversity the Whittaker distance matrix was calculated based on relative abundances with betadisper() function. To compare any differential effects from treatments an ANOVA was performed for richness and diversity. For ordination analysis, the dissimilarity matrix based on Bray-curtis distances was also calculated with relative abundances and two different fitting model analyses were applied. The function envfit() which fits centroids of class variable levels defined as a factor onto an ordination, and anosim() function for the analysis of similarities (ANOSIM). Finally, differential abundance analysis was performed with the OTU and taxa relative abundances under a zero-altered negative binomial or negative binomial model with pscl (Zeileis et al., 2008) and mass packages (Venables & Ripley, 2002), respectively, and corrected by false-discovery rate (FDR).

5.3 Results

In both studies, animals showed a good health status at the arrival with most of the animals adapting to facilities and feed positively. Both challenges promoted a clinical course of diarrhoea in most of the animals recovering spontaneously at the end of the study. The course of diarrhoea was more acute for the *Salmonella* challenge than for the ETEC F4 challenge and some casualties were registered in both trials. After the *Salmonella* challenge, one animal from DIC group was found dead at day 4 PI and two animals from DIC or CTR at day 6 PI. Within the ETEC F4 challenge, one animal from CTR was found dead at day 1 PI and a second animal form DIC group had to be euthanised following the clinical flowchart at day 3 PI. No statistical differences were seen in the number of causalities between the treatments (*P* >0.05) in any of the trials.

Animal performance

Effects of the experimental treatments on animal performance are shown in Table 5.2.

Table 5.2 Effect of the experimental treatments on growth performance in *Salmonella* and ETEC F4 trials.

	***Salmonella* trial**				**ETEC F4 trial**			
	CTR	**DIC**	**RSE**	***P***	**CTR**	**DIC**	**RSE**	***P***
BW (kg)								
Initial	8.11	8.12	0.143	0.900	5.62	5.56	0.098	0.239
Final	10.83	9.93	0.123	0.164	7.77	7.56	0.692	0.553
ADFI (g/day)								
Pre-inoculation[A]	233	226	28.1	0.598	178	162	38.4	0.431
0-4 PI[B]	218	193	46.7	0.302	219	211	45.8	0.729
4-8 PI[C]	428	443	79.5	0.713	282	276	82.0	0.894
Overall[D]	285	278	41.5	0.766	221	211	46.6	0.664
ADG[G] (g/day)								
Pre-inoculation[A]	122	109	60.7	0.675	77	78	41.6	0.986
0-4 PI[B]	-4	-38	108.7	0.541	129	117	90.5	0.796
4-8 PI[C]	326	316	162.6	0.903	260	164	80.6	0.032
Overall[D]	144	126	70.9	0.616	140	111	43.3	0.207

[A] Pre-inoculation: Period from 0 – 7th day before inoculation or 0 – 6th day before inoculation in *Salmonella* and ETEC F4 trials, respectively. [B] 4 PI: Period from 0 – 4th day post-inoculation. [C] 8 PI: Period from 4 – 8th post-inoculation. [D] Overall: Period from experimental day 1 – 14th or 1 – 15th in *Salmonella* and ETEC F4 trials, respectively. Legend: BW, body weight; ADFI, average daily feed intake; and ADG, average daily gain.

No significant effects were seen in final BW, ADFI or ADG with the administration of DIC in any of the trials except a decrease in the ADG (*P* = 0.032) in the second period PI (4-8 days PI) for DIC in the ETEC F4 trial.

Clinical signs

The evolution of the faecal consistency of the animals along the post-inoculation (PI) period is presented in Figure 5.1. After the oral challenge, faecal consistencies were impaired in both trials. The inoculation of *Salmonella* derived in more evident liquid diarrhoea (P_{day} <0.001) with scores reaching level 3 in most of the animals at day 2 PI. However, ETEC inoculation (P_{day} <0.015) only promoted clinical diarrhoea in a limited number of animals (hardly reaching scores of 1.8). No significant differences were seen related to the experimental diets in any of the trials.

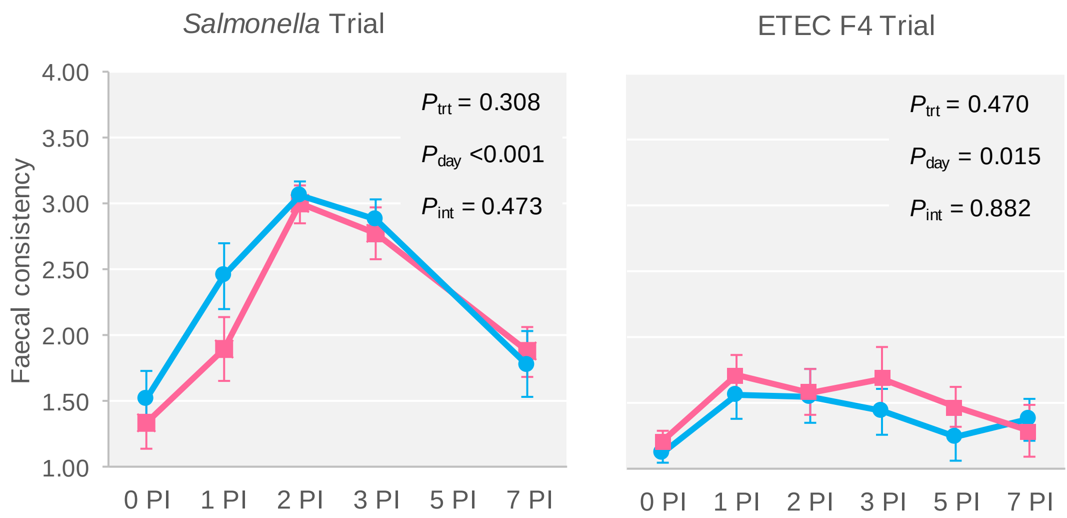


Figure 5.1 Evolution of faecal scores along the post-inoculation (PI) period in *Salmonella* and ETEC F4 trials by experimental group CTR (●), and DIC (■). Faecal score measured using a scale from 0 (hard and cloddy) to 4 (watery or with blood). Legend: P_{trt}, *P* value for DIC effect; P_{day}, *P* value for day effect; and P_{int}, *P* value for interaction effect (DIC x day).

The *Salmonella* challenge increased rectal temperature (40.6 ± 0.27 ºC on day 2 PI vs. 39.4 ± 0.22 ºC on day 0 PI; P_{day} <0.001) while ETEC F4 challenge did not affect this variable (39.1 ± 0.35 ºC on day 2 PI vs. 39.0 ± 0.24 ºC on day 0 PI; P_{day} = 0.21). In relation to the effect of diets, DIC had no significant influence on this variable (*P* >0.6).

Inflammatory response

Comparing trials, the response of the pro-inflammatory cytokine TNF-α and the acute-phase protein Pig-MAP was different to each pathogen. The *Salmonella* challenge promoted a more pro-inflammatory state (day 4 PI) giving higher values of TNF-α (320 ± 178.6 pg/mL and 139 ± 47.68 pg/mL for Trial 1 and 2, respectively) as well as higher levels of Pig-MAP (2.77 ± 2.252 mg/mL and 1.62 ± 1.339 mg/mL for Trial 1 and 2, respectively). As expected, towards day 8 PI, TNF-α (150 ± 60.6 pg/mL and 114 ± 19.96 pg/mL for Trial 1 and 2, respectively) and Pig-MAP concentrations (1.08 ± 0.667 mg/mL and 1.35 ± 1.421 mg/mL for Trial 1 and 2, respectively) were decreased and differences between trials were reduced. We were not able to detect statistical differences related to the experimental diets in any of the variables in any sampling day.

Microbiological analysis

In *Salmonella* trial, none of the animals seeded *Salmonella* in faeces on arrival and neither after the 6 days of adaptation. Serological analysis from day 4 PI confirmed that animals had not been exposed to *Salmonella* prior to the inoculation. Only one piglet per group (CTR and DIC) seroconverted at day 8 PI by applying a cut-off of Optical Density percentage (OD %) ≥40. Regarding the pathogen loads, all animals became positive but not all seeded countable numbers of the pathogen (>10^3 CFU/g fresh matter). Supplementation with DIC reduced the number of animals with countable numbers in caecum content particularly at day 8 PI (Table 5.3).

Table 5.3 Effect of the experimental treatments on the number of animals with countable numbers of *Salmonella* spp. (>10^3 log CFU/g FM) in different days PI in the *Salmonella* trial.

	CTR	**DIC**	***P***
Faeces			
1 PI	7/8	8/8	0.228
3 PI	8/8	6/8	0.080
7 PI	3/8	2/8	0.589
Caecum			
4 PI	8/8	5/7	0.065
8 PI	7/8	3/8	0.033

Figure 5.2 shows the different levels of excretion of *Salmonella* spp. in faeces and caecal content for the experimental diets. Although differences did not reach statistical significance, it was found a trend for DIC to increase *Salmonella* counts in faeces shortly after the inoculation (day 1 PI, *P* = 0.10; and day 3 PI *P* = 0.09) but to clear the pathogen more efficiently from caecum at day 4 PI compared to CTR (*P* = 0.07). At the end of the study (day 8 PI), it was also found an increase in the number of animals with unquantifiable counts (very low) in caecum with the DIC treatment (63 % vs. 12 % for DIC and CTR, respectively).

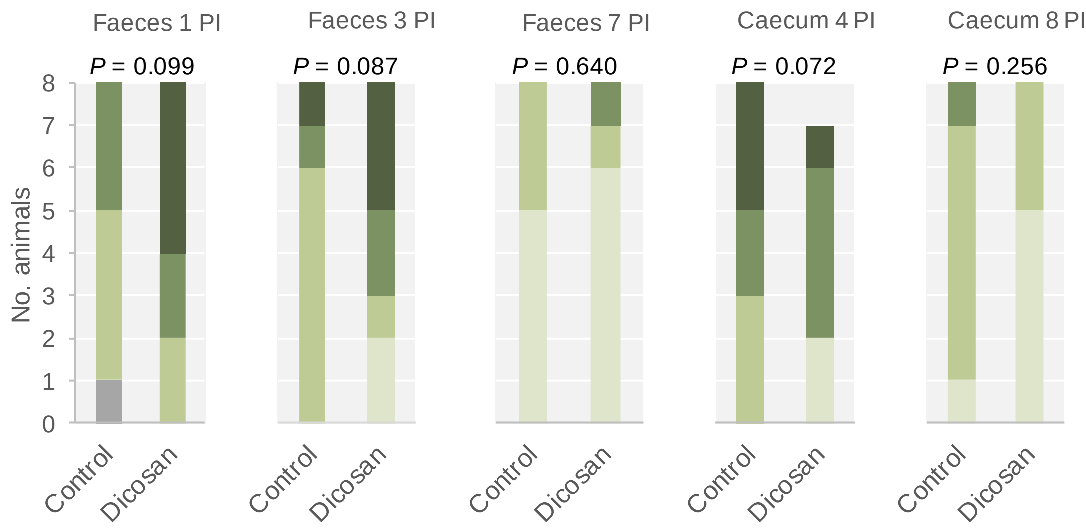


Figure 5.2 Number of animals with different range levels of *Salmonella* spp. counts in faecal samples at days 1, 3 and 7 PI and caecal content at days 4 and 8 PI (*Salmonella* trial). Range levels: Negative (0 CFU/g, ■), very low (<10^3 CFU/g, ■), low (10^3 - 10^4 CFU/g, ■), high (10^5 - 10^6 CFU/g, ■), and very high (10^7 - 10^8 CFU/g, ■).

Regarding the ETEC F4 trial, no significant differences were seen related to the experimental trials on enterobacteria nor coliform plate counts from faeces at arrival or after the week of adaptation (data not shown). Table 5.4 shows the microbiological analysis at days 4 and 8 PI of ileal and colonic digesta and also of ileal mucosa scrapes. At day 4 PI, counts of both bacterial groups were not different between diets. However, at day 8 PI, animals receiving DIC consistently had reduced numbers of enterobacteria and coliforms in ileum and colon content compared to CTR. This effect was especially manifested in ileum, with decreases of more than one log unit 5.38 vs. 6.71 log CFU/g FM (*P* <0.004) and 5.33 vs. 6.51 log CFU/g FM (*P* = 0.05) for enterobacteria and coliforms,

respectively. Attached enterobacteria in ileal mucosa trended also to be reduced with DIC at day 8 PI (*P* = 0.08).

Table 5.4 Effect of the experimental treatments on microbial plate counts on days 4 and 8 PI in the ETEC F4 trial.

		CTR	DIC	RSE	*P*
Ileum mucosa	Enterobacteria (log CFU/g FM)				
	4 PI	5.94	6.32	0.860	0.383
	8 PI	6.17	5.16	0.786	0.082
	Coliforms (log CFU/g FM)				
	4 PI	5.53	5.47	1.117	0.921
	8 PI	5.48	5.09	1.093	0.489
Ileum digesta	Enterobacteria (log CFU/g FM)				
	4 PI	6.81	6.76	0.616	0.872
	8 PI	6.71	5.38	0.783	0.004
	Coliforms (log CFU/g FM)				
	4 PI	6.02	6.08	0.923	0.889
	8 PI	6.51	5.33	0.829	0.013
Colon digesta	Enterobacteria (log CFU/g FM)				
	4 PI	10.70	9.91	1.161	0.213
	8 PI	10.85	9.90	1.028	0.083
	Coliforms (log CFU/g FM)				
	4 PI	8.94	8.92	0.992	0.974
	8 PI	10.81	9.77	0.980	0.052

Regarding the pathogen, it was not always present at quantifiable levels (limit of detection 3.83 log F4 gene copies/g FM). A slight trend was found between diets at day 4 PI in the prevalence of the pathogen (*P* = 0.10) with all CTR animals being positive to *E. coli* F4 in colon digesta (8/8 animals) but not all from DIC treatment (6/8 animals). At day 8 PI, pathogen presence was reduced in both groups with no significant differences between them (*P* = 0.30) (2/8 vs. 4/8 animals for CTR and DIC, respectively).

Microbiota 16S rRNA gene analysis

With the objective of assessing changes promoted by the additives in the intestinal microbiota, we analysed colonic digesta samples taken at day 8 PI by high-throughput sequencing (HTS) of the V3-V4 regions of the 16S rRNA gene.

The microbiota analysis was performed on a more conservative approach by using the OTU core, including only those species shared between experimental treatments, within

each experiment (836/843 OTUs in Trial 1 and 833/848 OTUs in Trial 2). Regarding the evenness of the reads, similar values were obtained comparing treated groups to CTR ones (*P* >0.34) (4.94 ± 0.375 and 4.82 ± 0.243 log10 no. reads/sample in Trial 1 and Trial 2, respectively). Chao1 index did not differ either between groups (*P* >0.74).

Regarding microbial diversity or structure, DIC administration did not alter neither alpha nor beta diversity (Whittaker index) after the *Salmonella* inoculation but did change the Bray-curtis distances (microbial structure) after the ETEC F4 inoculation as observed in Figure 5.3 in NMDS results (P_{envfit} = 0.035 and P_{ANOSIM} = 0.177).

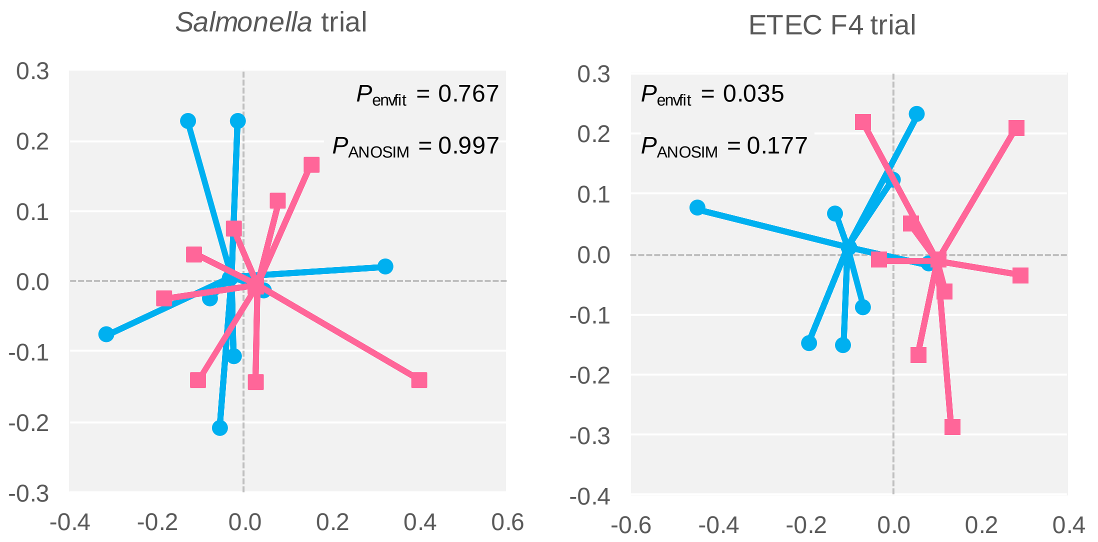


Figure 5.3 Non-metric dimensional scaling (NMDS) plot of dissimilarity matrix based on Bray-curtis distance clustered by experimental diets CTR (●), and DIC (■) for each challenge.

Focusing on aggregated taxa counts, Firmicutes and Bacteroidetes were the two major phyla (≥40 %), and Proteobateria following thereafter (7.3 ± 4.25 % in *Salmonella* trial and 9.1 ± 5.30 % in ETEC F4 trial). The ratio Firmicutes:Bacteroidetes (F/B) showed a numerical trend to decrease (*P* = 0.132) in *Salmonella* trial with DIC (1.14 vs. 1.47 in CTR group) but not in ETEC F4 trial (1.23 vs. 1.05 in CTR group, *P* = 0.335). Few minor phyla (<1 %), Elusimiocrobia, Fibrobacteres, Lentisphaerae, and Verrumicrobia, were encountered in *Salmonella* challenged animals but not in ETEC F4 ones. The majority of families observed in both trials were by order of abundance Prevotellaceae (27.9 ± 10.68 % and 29.0 ± 7.52 % in Trial 1 and 2, respectively), Veillonellaceae (22.7 ± 9.55 % and 15.4 ± 5.32 % in Trial 1 and 2, respectively), Rumminococaceae (11.4 ± 3.42 % and 11.4 2.63 % in Trial 1 and 2,

respectively) and Lachnospiraceae (8.29 ± 3.555 % and 9.25 ± 3.307 % in Trial 1 and 2, respectively) being detected 41 and 42 different families per trial, respectively, and representing around a 5 % the sum of least abundant ones (<1 %, 31 families per trial). Among genera, *Prevotella* genus was the major one (28.0 ± 10.7 % and 29.0 ± 7.52 % in Trial 1 and 2, respectively). Between the 63 genera detected, not all were in both trials (55 in *Salmonella* and 54 genera in ETEC F4 trials), and 40 and 42 genera were underrepresented (<1 %) in Trial 1 and 2, respectively, summing up to around 8 % of the total abundance. Unknown taxa represented the 7.2 ± 2.87 % and 6.7 ± 2.05 % of the families in *Salmonella* and ETEC F4 trials, respectively, whereas the 24.8 ± 6.63 % and 27.2 ± 4.72 % of the genera, respectively.

Regarding the influence of DIC on specific populations, in Figure 5.4 are shown the changes promoted by DIC addition in *Salmonella* and ETEC F4 trials, considering only taxa found in at least half of the samples. At phylum level, only the minor phylum Fibrobacteres (<1 %) was incremented in DIC supplemented animals compared to CTR (*P* = 0.02) under the *Salmonella* challenge. In parallel, the corresponding family and genus, Fibrobacteraceae and *Fibrobacter*, respectively, were enhanced in DIC animals (*P* <0.05). Other changes in *Salmonella* trial were also observed in minor families (<1 %) such as the reductions of the candidate Barnesiellaceae and also in genera such as *Dialister*, *Lachnobacterium* and *Butyrivibrio* (*P* <0.10). Although not significant, it was also relevant the numerical decrease registered on Enterobacteriaceae family in DIC animals compared to CTR under the *Salmonella* challenge (*P* = 0.16). When focusing on populations under the ETEC F4 challenge (Figure 5.4), few changes were observed with DIC. *Dialister* (P = 0.008) was significantly increased and *Lachnospira* tended to be reduced (*P* = 0.069). Other bacteria were numerically altered (*P* <0.20) by DIC representing most of them <1 % with exception of Veillonellaceae (*P* = 0.136) (around 15 %) and the genus within *Megasphaera* (*P* = 0.149) (around 5 %).

***Salmonella* trial**

Taxonomic level	Taxon	Ln change (DIC vs. CTR)	adjusted *P*
Phylum	Fibrobacteres		0.019
Family	candidate Barnesiellaceae	-4.499	0.003
	Fibrobacteraceae		0.024
	Enterobacteriaceae		0.157
	Spirochaetaceae		0.192
	Alcaligenaceae		0.192
Genus	*Dialister*		0.000
	Fibrobacter		0.033
	Lachnobacterium		0.035
	Butyrivibrio		0.095

ETEC F4 trial

Taxonomic level	Taxon	Ln change (DIC vs. CTR)	adjusted *P*
Family	Veillonellaceae		0.136
	Oxalobacteraceae		0.151
	Deferribacteraceae		0.151
	Turicibacteraceae		0.176
Genus	*Dialister*	5.981	0.008
	Lachnospira		0.069
	Megasphaera		0.149
	Coprococcus		0.149
	Oxalobacter		0.173
	Mucispirillum		0.173
	Turicibacter		0.183

Figure 5.4 Ln changes promoted by DIC supplementation (adjusted *P* <0.20) in taxa under *Salmonella* challenge or ETEC F4 challenge. Positive values (■) and negative values (■) indicate greater and lower abundance, respectively, in treated animals. Taxa are sorted by level of significance (from higher to lower). Only extreme positive and negative ln changes are displayed as reference. Differences presented are based only on taxa detected in at least half of the samples per diet.

When looking at changes produced at OTU level, i.e. species, only those found in at least half of the samples were considered. In Figure 5.5 are presented the differences promoted by DIC (*P* <0.05) under the *Salmonella* and ETEC F4 challenges. As observed in aggregated taxa, in *Salmonella* trial several OTU assigned to Lachnospiraceae (new refs. OTU-2808 and 2343, and OTU-296082) were reduced in DIC animals, including *Lachnobacterium* genus (OTU-584463). In addition, a Spirochaetes associated OTU was enhanced in DIC animals (OTU-300859) and also, several OTU associated to *Prevotella* (within Bacteroidetes) were increased in DIC animals possibly explaining the reduction of the F/B ratio mentioned before. Nonetheless, other OTU also corresponding to Bacteroidetes were reduced, what reminds us the high variability within a taxonomic group and the importance of considering the strain.

***Salmonella* trial**

CTR	DIC	OTU code	Taxonomic classification
0.480	0.701	300859	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella*
0.273	0.175	349257	Firmicutes; Clostridiales; Lachnospiraceae; *Lachnospira*
0.005	0.089	New ref. 2497	Firmicutes; Clostridiales
0.005	0.062	New ref. 6586	Unassigned
0.055	0.002	225636	Firmicutes; Erysipelotrichales; Erysipelotrichaceae
0.048	0.003	315846	Bacteroidetes; Bacteroidales; cand. Barnesiellaceae
0.039	0.015	535375	Bacteroidetes; Bacteroidales; Bacteroidaceae; *Bacteroides ovatus*
0.065	0.052	New ref. 6366	Bacteroidetes; Bacteroidales; cand. Paraprevotellaceae; cand. *Prevotella*
0.001	0.024	410242	Firmicutes; Clostridiales; Christensenellaceae
0.003	0.017	1832447	Spirochaetes; Sphaerochaetales; Sphaerochaetaceae; *Sphaerochaeta*
0.003	0.025	New ref. 2311	Bacteroidetes; Bacteroidales
0.012	0.033	357471	Firmicutes; Clostridiales
0.008	0.033	New ref. 1872	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella stercorea*
0.009	0.022	New ref. 1748	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella*
0.008	0.023	296082	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella*
0.017	0.001	584463	Firmicutes; Clostridiales; Lachnospiraceae; *Lachnobacterium*
0.001	0.008	New ref. 2808	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella stercorea*
0.002	0.009	New ref. 648	Bacteroidetes; Bacteroidales; cand. Paraprevotellaceae; cand. *Prevotella*
0.025	0.021	302538	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella stercorea*
0.014	0.013	526773	Firmicutes; Clostridiales; Lachnospiraceae; *Blautia*

ETEC F4 trial

CTR	DIC	OTU code	Taxonomic classification
2.430	1.654	524371	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella stercorea*
0.210	0.261	584083	Firmicutes; Clostridiales
0.038	0.220	298050	Firmicutes; Clostridiales; Veillonellaceae; *Megasphaera*
0.032	0.318	344804	Firmicutes; Clostridiales; Lachnospiraceae; *Coprococcus*
0.001	0.056	403701	Firmicutes; Clostridiales; Veillonellaceae; *Dialister*
0.006	0.084	513445	Bacteroidetes; Bacteroidales; Bacteroidaceae; *Bacteroides*
0.049	0.046	248447	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella*
0.004	0.040	844589	Bacteroidetes; Bacteroidales; S24-7
0.034	0.030	289468	Bacteroidetes; Bacteroidales; cand. Paraprevotellaceae; YRC22
0.021	0.014	New ref. 2220	Bacteroidetes; Bacteroidales; Prevotellaceae; *Prevotella*
0.026	0.026	287798	Firmicutes; Erysipelotrichales; Erysipelotrichaceae
0.017	0.011	264967	Firmicutes; Clostridiales; Veillonellaceae; *Megasphaera*
0.008	0.004	New ref. 5084	Bacteroidetes; Bacteroidales; cand. Paraprevotellaceae; cand. *Prevotella*
0.010	0.011	798164	Firmicutes; Clostridiales
0.008	0.013	583134	Firmicutes; Clostridiales; Ruminococcaceae
0.399	0.140	843553	Firmicutes; Clostridiales; Lachnospiraceae; *Lachnospira*
0.282	0.000	366623	Firmicutes; Clostridiales; Lachnospiraceae; *Coprococcus*
0.143	0.081	826624	Firmicutes; Clostridiales

Figure 5.5 Heatmap of differential abundant OTUs (relative abundance and adjusted *P* value <0.05) between DIC and CTR groups in each pathogen challenge: *Salmonella* trial; and ETEC F4 trial. Relative abundance from 0 % (■) to 2.5 % (■). Only OTUs present in at least half of the animals per diet were evaluated.

In ETEC F4 trial, increases of OTU assigned to Veillonellaceae family and *Dialister* (OTU-403701) or *Megasphaera* (OTU-298050 and 264967) genera were also observed whereas

OTUs from genera such as *Lachnospira* (OTU-843553) and *Coprococcus* (OTU-344804 and 366623) were reduced by DIC as shown in aggregated taxa. Likewise, as well as genus *Turicibacter* was seen reduced before, at OTU level an OTU from a related family, Erysipelotrichaeceae (Kanno et al., 2015), was also reduced by DIC (OTU-287798). Regarding OTU assigned to *Prevotella* (OTU-248447, 524371, and new ref. OTU-2220), in the ETEC F4 trial they were reduced by DIC contrary to what was observed under the *Salmonella* challenge.

Fermentation

Table 5.5 shows the modifications on pH and the main fermentation products in ileum and colon digesta for both trials. Ileal pH was not significantly modified by the experimental treatments except for a numerical reduction (7.00 vs. 7.20; P = 0.11) observed for DIC at day 4 PI in the *Salmonella* trial. In ileum, the major product of fermentation was lactic acid ranging 1.80-117 and 1.98-105 mmol/kg in Trial 1 and 2, respectively. No differences were detected between diets. Acetic acid in ileum (data not shown) was found at very low levels in both trials (average values of 1.61 mmol/kg and 4.44 mmol/kg for Trial 1 and 2, respectively) with several animals below the minimum level of detection at day 8 PI (12 for Trial 1 and 3 for Trial 2) and no differences between experimental diets.

Regarding colon digesta, no obvious changes were found in pH despite a slight reduction with DIC at day 4 PI in the *Salmonella* trial (6.70 vs. 6.92; P = 0.14). Ammonia was not modified by the experimental diets although average mean values were lower in the Trial 2 compared to Trial 1 (10.7 vs. 28.4 mM at day 4 PI and 18.0 vs. 48.2 mM at day 8 PI). Total SCFA was neither modified by the experimental diets but showed an increase from day 4 to 8 PI in both Trials (from 92.7 to 141 and 104 to 159 mmol/kg, respectively). The fermentation profile showed little changes with the diets and it was only detected an increase in the valeric acid proportion in colon with DIC at day 8 PI in ETEC trial (P = 0.03) showing a similar numerical trend in *Salmonella* trial at day 4 PI (P = 0.12).

Table 5.5 Effects of experimental treatments on intestinal fermentation on days 4 and 8 PI in *Salmonella* and ETEC F4 trials.

		***Salmonella* trial**				**ETEC F4 trial**			
		CTR	**DIC**	**RSE**	***P***	**CTR**	**DIC**	**RSE**	***P***
Ileum	pH								
	4 PI	7.20	7.00	0.228	0.111	6.81	6.80	0.208	0.891
	8 PI	7.37	7.34	0.190	0.786	6.86	6.77	0.195	0.346
	NH_3[A] (mM FM)								
	4 PI	4.35	2.01	2.773	0.113	0.63	1.44	1.907	0.431
	8 PI	14.56	19.08	8.888	0.326	1.01	1.08	0.387	0.752
	Lactic acid (mM FM)								
	4 PI	10.5	29.8	29.17	0.207	23.2	27.7	18.91	0.652
	8 PI	41.8	23.4	27.09	0.196	19.1	36.0	25.73	0.209
Colon	pH								
	4 PI	6.93	6.70	0.274	0.137	6.26	6.37	0.238	0.360
	8 PI	7.07	7.04	0.156	0.671	5.97	6.07	0.266	0.464
	NH_3[A] (mM FM)								
	4 PI	32.5	24.4	19.94	0.431	10.7	10.8	5.821	0.969
	8 PI	57.3	39.1	25.86	0.180	18.5	17.6	6.763	0.801
	Total SCFA (mM FM)								
	4 PI	98	88	37.1	0.613	107	102	21.5	0.606
	8 PI	147	136	17.6	0.244	155	163	21.5	0.461
	Acetic acid[A] (%)								
	4 PI	55.8	55.2	8.22	0.892	60.5	60.1	6.01	0.907
	8 PI	56.0	54.2	7.37	0.629	61.3	60.2	4.06	0.573
	Propionic acid[A] (%)								
	4 PI	26.6	24.6	3.86	0.317	22.5	24.7	5.91	0.479
	8 PI	21.7	25.9	6.52	0.218	22.6	23.1	3.32	0.778
	Butyric acid[A] (%)								
	4 PI	12.9	14.3	6.01	0.649	12.6	11.2	3.78	0.492
	8 PI	17.0	15.3	3.61	0.373	13.5	12.7	2.09	0.452
	Valeric acid[A] (%)								
	4 PI	2.65	4.10	1.804	0.118	2.73	2.55	1.327	0.802
	8 PI	4.10	3.53	1.467	0.456	1.59	2.84	1.056	0.033
	BCFA[A] (%)								
	4 PI	2.16	1.83	1.186	0.586	1.68	137	0.774	0.465
	8 PI	1.27	1.11	0.531	0.566	0.95	1.21	0.365	0.176

[A] Each SCFA molar proportion relative to total SCFA. Legend: NH_3, ammonia; SCFA, short-chain fatty acids; and BCFA, branched-chain fatty acids.

Histomorphometry

Table 5.6 shows changes promoted in ileal histomorphometry by the experimental diet. Villus height showed a marked increase from day 4 to 8 PI in both trials, but it was not modified by the experimental diets and neither the crypt depth or the villus:crypt ratio. The number of mitoses were remarkably high at day 4 PI in the *Salmonella* trial decreasing

to levels similar to those found in the ETEC F4 trial at day 8 PI. No differences were detected related to the inclusion of DIC neither in this variable nor in the number of goblet cells. Intriguingly, it was found a different effect of DIC in the villous intraepithelial lymphocytes (IEL) at day 8 PI depending on the trial. Whereas IEL counts trended to decreased with DIC in *Salmonella* trial (*P* = 0.07), they were increased (*P* = 0.08) with the additive in the ETEC F4 trial. In addition, at day 8 PI whereas IEL were remarkable higher in the animals receiving the CTR diet in Trial 1 compared to those in Trial 2 (3.92 vs. 2.39 cells/100 µm), values were similar for the animals receiving DIC in both trials (3.06 and 2.88 cells/100 µm for Trial 1 and 2, respectively).

Table 5.6 Effect of experimental treatments on ileal histomorphometry on days 4 and 8 PI in *Salmonella* and ETEC F4 trials.

	***Salmonella* trial**				**ETEC F4 trial**			
	CTR	**DIC**	**RSE**	***P***	**CTR**	**DIC**	**RSE**	***P***
Villus height (µm)								
4 PI	185	183	65.5	0.962	278	229	89.4	0.289
8 PI	282	276	58.1	0.852	323	321	63.7	0.955
Crypt depth (µm)								
4 PI	315	323	69.6	0.815	196	182	30.9	0.380
8 PI	300	290	37.1	0.596	189	184	19.7	0.600
Villus:Crypt ratio								
4 PI	0.600	0.627	0.2573	0.841	1.47	1.29	0.469	0.454
8 PI	0.985	0.993	0.2089	0.944	1.78	1.79	0.364	0.935
MIT (cells/100 µm)								
4 PI	0.251	0.325	0.1597	0.390	0.078	0.068	0.1031	0.843
8 PI	0.075	0.099	0.0823	0.569	0.104	0.091	0.1414	0.862
GC (cells/100 µm)								
4 PI	2.20	1.93	0.851	0.556	1.75	1.43	0.542	0.262
8 PI	1.49	1.28	0.548	0.457	1.31	1.33	0.519	0.954
IEL (cells/100 µm)								
4 PI	2.92	2.76	1.509	0.841	2.07	2.04	0.482	0.917
8 PI	3.92	3.06	0.882	0.072	2.39	2.88	0.523	0.080

Legend: MIT, mitoses in crypts; GC, goblet cells in villi; and IEL, intraepithelial lymphocytes in villi.

5.4 Discussion

The present study aimed to evaluate the possible antimicrobial effect from a mixture of salts of medium-chain fatty acids (MCFA) from coconut distillates (DIC) against digestive pathogens such as *Salmonella* Typhimurium or ETEC F4 and the consequent reinforcement of gut health and general health status of early-life weaned piglets. In order to determine these objectives, DIC was administered in-feed in two different trials,

each with one of the two pathogens. In both trials, animals exhibited signs of diarrhoea after the oral challenge and an immediate decrease in the feed intake. However, after the *Salmonella* challenge, the response of the animals was more acute, compared to ETEC F4, with a more liquid but self-limiting diarrhoea, fever and increased levels of Pig-MAP and TNF-α (almost doubled at day 4 PI) compared to those from animals inoculated with ETEC F4. *Salmonella* challenged animals also had shorter ileal villi, despite being one week older, and higher number of IEL in ileum compared to ETEC F4. Previous works have displayed similar outcomes (Jensen et al., 2006; Spitzer et al., 2014; Barba-Vidal et al., 2017a). Actually, both pathogens display different pathogeneses. *Salmonella* is an intracellular and invasive pathogen that has de capability to translocate the epithelial barrier and reach the lymph nodes (Martín-Peláez et al., 2010); while *E. coli* F4 attaches to the intestinal mucosa and secretes toxins leading to malabsorption (Fairbrother & Gyles, 2012). This could explain the higher recruitment of IEL after the *Salmonella* challenge, most assuredly involved in a more severe pathogenesis compared to ETEC F4.

During the week of adaptation after weaning, animals from the different diets displayed similar performances with no influence registered for DIC supplementation. Other authors, however, have described improvements in performance related to the supplementation of MCFA. Some works evaluating MCFA as an energy source, at higher doses (2.1-7.75 %), registered a growth improvement (Cera et al., 1990; Rodas & Maxwell, 1990; Li et al., 2015; Weng et al., 2017) that could be justified by the more rapid digestion and absorption of these fatty acids. Improvements have also been seen in animals challenged with ETEC F4 (Lei et al., 2017). Regarding possible effects of the MCFA supplementation on feed intake, we neither detected any impact of the experimental diets. The potential impact of MCFA as additives on feed intake is controversial. When supplemented at high levels, as a rapid source of energy, MCFA could prompt satiety due to their rapid oxidation in liver (Guillot et al., 1993; Geng et al., 2016b). Moreover, MCFA have also been described as rancid and to present bad palatability (Decuypere & Dierick, 2003; Desbois & Smith, 2010), what could therefore decline the feed intake and impair the adaptation to dry feed after weaning. Nonetheless, in our study no negative impact of DIC was registered in the feed intake during the first week post-weaning. The lower

dosage used (at 0.3 %) compared with other authors, and the different nature and composition of MCFA blends, could explain the discrepancies with other studies.

One of the most remarkable effects of this study was the specific antimicrobial effects of supplemented MCFA on populations of *Salmonella*, coliforms and enterobacteria. *Salmonella* spp. counts in caecum trended to be reduced by DIC despite a higher initial colonisation after the challenge. After the ETEC F4 inoculation, DIC consistently diminished enterobacteria and total coliforms in luminal contents of ileum and colon (particularly at day 8 PI) and the same trend was observed in ileal mucosa, despite effects on the luminal *E. coli* F4 pathogen were small. As stated in the introduction, a higher antibacterial activity has been described for MCFA compared to other free fatty acids (Batovska et al., 2009). Most recent studies have demonstrated *in vitro* activity against a wide range of pathogenic bacteria (Batovska et al., 2009; Manohar et al., 2013; Shilling et al., 2013; Vande Maele et al., 2016). Lauric acid (C12) has been shown to have the highest antimicrobial activity among free fatty acids (Skrivanová et al., 2006; Tangwatcharin and Khopaibool, 2012; Shilling et al., 2013), being the major compound of the distillates of coconut (48.4 % of crude fat, CF). Regarding *in vivo* studies evaluating the effect of MCFA administration on pathogens, some authors have described decreased numbers of clostridia or *E. coli* (Dierick et al., 2004; Hanczakowska et al., 2011; Upadhaya et al., 2014; Kuang et al., 2015; Upadhaya et al., 2018) and also *Salmonella* (Rasschaert et al., 2016). These effects reported on the colonisation of the gut by different pathogens could had been mediated by a direct antimicrobial effect of MCFA but also indirectly by changes promoted in the intestinal microbiota by these additives. In this regard, different *in vivo* trials in pigs have described significant changes on bacterial populations promoted by MCFA supplementation (Zentek et al., 2012; Zentek et al., 2013). Variability of these effects found in the literature could be the result of differences between MCFA sources, blends, dosages and ways of administration, making difficult the direct comparison between works. Particularly, most of the experimental blends tested were composed of a combination of caprylic (C8) and capric (C10) acids and negligible quantities of lauric acid (C12) or their conjugated forms into triacylglycerol (MCT) with the need of lipases to release, not always successfully, the free fatty acids.

Up to our knowledge, very few works have evaluated the effect of MCFA on intestinal microbiota performing the entire profiling of their populations and particularly in front of a pathogen challenge. Results of our work showed that, in global terms, the microbiota profiles observed after the two challenges were not substantially different from those previously described for piglets of this age (Xiao et al., 2016; Holman et al., 2017), suggesting that these animals did not suffer any drastic dysbiosis. Actually, other challenges in piglets with *Salmonella* could not differentiate clusters from unchallenged and *Salmonella* challenged animals either (Borewicz et al., 2015) and even were not able to detect *Salmonella* genus by the HTS, as occurred in our animals. In this regard, (Bearson et al., 2013) observed that the *Salmonella* challenge triggered a change in specific populations rather in structure, depending on the level of colonisation.

Regarding changes promoted by DIC diets in microbiota, we observed a differential response depending on the pathogen challenge. In the case of ETEC F4 trial, the challenge was weaker, and the impact of the MCFA supplementation was more evident at structural level (P_{envfit} = 0.035) than with *Salmonella*. In both trials, however, DIC diets were able to modify significantly particular microbial taxa, although the bacterial groups affected were different. Whereas within the ETEC F4 challenge the most relevant change was the significant increase of *Dialister* (P = 0.008), within Veillonellaceae family, with the *Salmonella* challenge the most relevant change was the significant increase registered in the Fibrobacteres phylum (P = 0.019). Fibrobacteres has been described to increase with the age of animals and also has been previously associated to lower colonised *Salmonella* profiles (Bearson et al., 2013). Accompanied with the (numerical) reduction of Enterobacteriaceae, it could be hypothesised that the supplemented MCFA would prompt a more fibrolytic-like adult microbial profile. Also, it is interesting to remark that within both challenges, several OTUs from the Lachnospiraceae family were decreased with the DIC diets and that *Lachnospira* genus has been reported to increase with *Salmonella* (Leite et al., 2018).

This differential response of microbiota to DIC supplementation depending on the pathogen challenge could also be associated to the different response registered in IEL at ileal level. Whereas at day 8 PI animals treated with DIC and challenged with *Salmonella* presented lower numbers of IEL than CTR group, when challenged with ETEC F4 IEL

numbers increased. A possible hypothesis would advocate that the pigs in DIC group from *Salmonella* trial would have retained a more benign environment within the gastrointestinal tract. On the other side, the reduction of possible specific commensal populations such as enterobacteria and coliforms in ETEC F4 challenged animals would have triggered a more hostile milieu, thus mobilising more IELs compared to CTR group. This fact could be related with a possible readaptation of the community structure as seen in the NMDS results. However, other mechanisms related with a direct modulator effect of MCFA on the immune response of the animal should not be discarded. MCFA supplemented to pre-weaning piglets as triglycerides (MCT) have shown to stimulate immune response in stomach regardless of the complexity of the microbiota (Trevisi et al., 2017). Another possible hypothesis is that in *Salmonella* trial, the clear reduction of the counts of the pathogen in caecum by DIC addition would have stopped the recruitment of IELs since these defence cells have been found nearby *Salmonella* pathogen (Hoytema van Konijnenburg et al., 2017). Actually, IEL were higher within the *Salmonella* challenge compared to ETEC F4 reflecting a more acute course of diarrhoea. If we consider that the IELs remain quiescent until real danger signals appear (Cheroutre, 2005), it seems reasonable that only after the *Salmonella* challenge, DIC treatment had the opportunity to show effects on the IEL response.

Trying to elucidate possible associations between microbial populations and gut variables assessed in this study, particularly those modified by the experimental treatments, we performed a correlation analysis. The most relevant correlations (r >0.5 and adjusted P <0.10) are presented in Table 5.7. Interestingly, candidate Barnesiellaceae and Enterobacteriaceae families were positively correlated with IEL counts in *Salmonella* trial whereas in ETEC F4 trial no significant correlation was found. This could be somehow related to the differential response registered in IEL with DIC between both trials. Regarding valeric acid, it showed only a significant high inverse correlation with Clostridiaceae (P = 0.011) in *Salmonella* trial. Under the ETEC F4 challenge, valeric acid was positively correlated with *Megasphaera* (r = 0.837) and the corresponding family Veillonellaceae (r = 0.784). This correlation is coincident with the higher presence of *Dialister* at day 8 PI in DIC animals and the higher percentage of valeric acid registered. *Dialister* and *Megasphaera*, among other members within the Veillonellaceae family,

have actually been reported to produce valeric acid as an end metabolite of fermentation of carbohydrates and lactate (Marchandin et al., 2010).

Table 5.7 Pearson's correlations (r) between IEL or valeric acid and bacterial populations ($r \geq 0.5$) for *Salmonella* and ETEC F4 trials.

Gut variable	Microbiota	Pearson (r)	adjusted P
***Salmonella* trial**			
IEL	candidate Barnesiellaceae	0.698	0.055
IEL	candidate Odoribacteraceae	0.694	0.057
IEL	*Odoribacter*	0.694	0.057
IEL	Enterobacteriaceae	0.686	0.065
Valeric acid	Clostridiaceae	-0.782	0.011
ETEC F4 trial			
Valeric acid	*Megasphaera*	0.837	0.005
Valeric acid	Veillonellaceae	0.784	0.023

5.5 Summary

In conclusion, the results obtained from this study support the activity of a blend of sodium salts of distillates of coconut oil in reducing the hindgut colonisation by pathogenic populations such as enterobacteria, *E. coli* and *Salmonella* in weaned piglets orally challenged. Effects could be mediated by the changes promoted in the microbiota ecosystem as significant effects were registered in different microbial groups. Under a *Salmonella* challenge, significant increases were registered in the Fibrobacteres phylum and after an ETEC F4 challenge the most relevant changes were registered in the *Dialister* genus of the Veillonellaceae family within a diverging microbial structure. It was also found a differential impact of this blend on intestinal local immune response depending on the pathogen, increasing ileal intraepithelial lymphocytes after the *Salmonella* challenge but decreasing after the ETEC F4 challenge. These differential effects of the supplemented MCFA could respond to different complex interactions between the opportunistic pathogens, the commensal microbiota and the host response.

Chapter 6 Impact of sodium butyrate or sodium heptanoate protected with medium-chain fatty acids on the health of weaned piglets challenged with ETEC F4

6.1 Introduction

Post-weaning diarrhoea (PWD) in piglets caused by the enterotoxigenic (ETEC) *Escherichia coli* deserves special attention due to its impact on growth, and morbidity causing important economic losses to the porcine sector (Heo et al., 2013). This problematic, in turn, brings an earlier and unacceptable prophylactic use of antimicrobials in the weaner stages that has had public concerns and deleterious consequences due to the raise of antimicrobial resistances and to cross-resistance with human medicine (Prigitano et al., 2018). Moreover, other antimicrobials such as zinc oxide (ZnO) might also have a negative environmental impact (Jondreville et al., 2003) and thereby a bad reputation.

In this context, modern and intensive farming systems require new management strategies to influence positively animal health and welfare and, in this way, help to reduce the use of antimicrobials. With this objective, a first approach was the ban of the use of antibiotic as growth promoters in Europe (R(CE) 1831/2003), and more recently worldwide the adoption of the Global Action Plan by WHO (2015). Within the main strategies, in front of possible further future bans and restrictions, the research on new bioactive in-feed ingredients appears as a promising alternative, even more especially if based on natural-source derived compounds for a wider acceptance.

Several strategies based on the in-feed addition of short- or medium-chain fatty acids have been tested, particularly in the weaned piglet, as a measure to keep the gut healthy and preventing PWD. Actually, short-chain fatty acids (SCFA) (Gaudier et al., 2004; Onrust et al., 2018) are naturally produced in the animal gut and are believed to control bacterial populations as well as interact with the host, being a source of energy for compromised animals. More recently, medium-chain fatty acids (MCFA) supplemented in piglet diets have also demonstrated to have antimicrobial/modulatory activities being capable of improving performance. Based on these properties, several natural sources of MCFA such are coconut or *Cuphea* seeds have been proposed as potential functional ingredients to be used in piglet diets (Dierick et al., 2004; DebMandal & Mandal, 2011; Li et al., 2015; Baltić et al., 2017).

Trying to get synergic benefits, several authors have tested combinations of different fatty acids (Kuang et al., 2015; Vande Maele et al., 2016), even sometimes combined with other additives as natural flavours or essential oils from plants extracts (Piva et al., 2007; Grilli et al., 2013). How they exert these effects, however, is still under review, and few studies address the underlying mechanisms. Some works comparing these alternative strategies to antibiotics (Kuang et al., 2015; Li et al., 2018) reported different influences on intestinal health in terms of microbiota ecosystem despite both strategies showed comparable benefits on growth and diarrhoea prevention.

In previous works of our group, we tested the potential of different in-feed additives to control different digestive pathogens in weaning piglets. Particularly, we tested fat-protected sodium butyrate or sodium heptanoate in front of an ETEC F4 challenge (Chapter 4) and also the potential of coconut distillates, consisting of MCFA, mainly C12, against ETEC F4 and also *Salmonella* (Chapter 5). Results from those studies demonstrated a different impact of each of these additives on the fermentation along the digestive tract, the colonic microbiota or the local intestinal immunity depending on the additive. The MCFA mix showed the clearest effect reducing pathogen loads in the gut. In the present work, we hypothesised that protecting sodium butyrate or sodium heptanoate with coconut distillates could improve synergistically their efficacy against digestive pathogens. Therefore, the objective of this study was to evaluate the effect of the two new developed additives consisting of salts of sodium butyrate or sodium heptanoate, protected with the coconut distillates, on the gut health of weaned piglets challenged with ETEC F4.

6.2 Material and methods

Two different experiments (Trial 1 and Trial 2) were performed to evaluate the effect of each of the two additives against an oral challenge with ETEC F4. Both trials were run at the Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (UAB) and received prior approval (permit no. CEAAH2933/HR-10-13) from the Animal and Human Experimental Ethical Committee of this institution. The treatment,

management, housing, husbandry and slaughtering conditions conformed to European Union Guidelines (Directive 2010/63/EU).

Animals and housing

The two trials followed a Level 2 – High Risk Biosecurity Procedures with appropriate training of the involved personnel. For both trials, 48 male piglets were used each from high sanitary status farms. [Landrace x Large White] x Piétrain piglets came from non-vaccinated mothers against *Escherichia coli* weaned at 21 days of age and 5.8 ± 0.57 kg and 5.6 ± 0.93 kg of body weight (BW), for the first (Trial 1) and the second (Trial 2) trials, respectively.

Piglets were transported to the UAB facilities and placed in 16 3-m^2-pens with three animals per pen. Each pen of three animals was readjusted by weight (a low-, an intermediate- and a high-weighed) to obtain a final homogenous weight among the pens. The experimental treatments were distributed evenly among the pens with eight replicates each (n = 8).

The weaning rooms were equipped with automatic heating and forced ventilation. The experiments were conducted during the spring and winter seasons (May and February – March, respectively) under a mean room temperature of 26.5 ± 1.29 ºC. Both trials were maintained under a 13 h light/11 h dark lighting regimen.

Experimental products, diets and feeding regime

The feed additives were supplied by Norel S.A (Madrid, Spain), consisting of two salts of organic acids protected with a mixture of sodium salts of distilled coconut MCFA. In Trial 1, sodium butyrate was protected with sodium salts of a distilled coconut MCFA mixture (50 % of butyrate) (Dicosan Plus), and in Trial 2, sodium heptanoate was similarly protected with the same sodium salts of a distilled coconut MCFA mixture (50 % of heptanoate) (Hept'on Plus). Both additives were added to the diets at 3 kg/t following the manufacturer's recommendations.

Diets (Table 6.1) were formulated to satisfy the nutrient requirement standards for pigs (NRC, 2012). A plain diet without additives (CTR) was manufactured in the same batch within each trial and the corresponding treatment, sodium butyrate or heptanoate both protected with the sodium salts of distilled coconut MCFA (BUT+ or HPT+, respectively), was included in a second mixture resulting in two experimental diets per trial.

Animals received the experimental diets *ad libitum* and had free access to water over the 16 days of the experiment.

Table 6.1 Ingredient composition and nutrient analysis of the basal diet as-fed basis (g/kg).

Ingredients			
Maize	280.7	Mono-calcium phosphate	21.3
Wheat	170.0	Calcium carbonate ($CaCO_3$)	8.2
Barley 2 row	150.0	L-Lysine HCL	4.5
Extruded soybean	122.4	Vitamin-Mineral Premix*	4.0
Sweet whey-powder (cattle)	100.0	Sodium chloride (marine salt)	3.0
Fishmeal LT	50.0	DL-Methionine 99	2.4
Soybean meal 47	50.0	L-Threonine	2.3
Whey powder 50 % fat	30.3	L-Tryptophan	0.9
Calculated nutrients			
Metabolisable energy (kcal/g)	3315.4	Digestible lysine	13.9
Crude protein	211.1	Digestible methionine plus cysteine	8.2
Calcium	6.5	Digestible threonine	9.0
Digestible phosphate	4.1	Digestible tryptophan	2.8
Chemical composition	**Trial 1**	**Trial 2**	
Dry matter	915.2	915.0	
Ash	66.8	48.1	
Crude fat	86.0	63.6	
Crude protein	187.2	196.4	
Neutral detergent fibre	81.3	82.0	
Acid detergent fibre	27.9	31.3	

*Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D3, 39.9 mg vitamin E, 3 mg vitamin K3, 2 mg vitamin B1, 2.3 mg vitamin B2, 3 mg vitamin B6, 0.025 mg vitamin B12, 20 mg calcium pantothenate, 60 mg nicotinic acid, 0.1 mg biotin, 0.5 mg folic acid, 150 mg Fe, 156 mg Cu, 0.5 mg Co, 120 mg Zn, 49.8 mg Mn, 2 mg I, 0.3 mg Se.

Bacterial strain

The bacterial strain used was an enterotoxigenic *Escherichia coli* (ETEC) F4 strain (positive to virulence factors F4ab, F4ac, LT, STb, and negative to EAST1 and F6, F18, F41, STa, VT1, VT2, and EAE) that was isolated from 14-week old pigs and provided by the Diseases

Laboratory of the UAB (ref. COLI30/14-3). The oral inocula were prepared by an overnight incubation at 37 ºC and 250 rpm in brain heart infusion (BHI; Laboratorios Conda S.A). To confirm the inocula concentrations, serial dilutions were cultured in Luria agar (LA) by an overnight incubation at 37 ºC. For the first trial, the final inoculum was 2.3 x 10^8 CFU/mL and for the second trial 2.5 x 10^8 CFU/mL.

Experimental procedure

At arrival and after 7 days of adaptation (before the challenge), faecal samples for microbiological analysis (enterobacteria and total coliform counts) were obtained aseptically after spontaneous defecation associated to the manipulation of the animal or by digital stimulation. Samples were taken from the heaviest-weighted piglet of each pen (N = 16).

After the week of adaptation, on day 8, a single oral dose of 6 mL of ETEC F4 (1.4 x 10^9 and 1.5 x 10^9 CFU for Trial 1 and 2, respectively) was administered to the animals. In order to ensure that the stomach was full at the time of inoculation, and facilitate bacterial colonisation, feed withdrawal was done the previous day at 21:00 and provided back 30 minutes before inoculation.

Individual body weight (BW) and pen feed intake were registered during the adaptation period, and further recorded on days 0, 4 and 8 post-inoculation (PI). After the oral challenge, faecal score was checked daily and clinical signs evaluated (i.e. dehydration, and apathy) always by the same person. Mortality was also registered, and no antibiotic treatment was administered to any of the animals on any of the trials. Faecal score was measured using a scale: 0 = hard, 1 = solid and cloddy, 2 = soft with shape, 3 = very soft or viscous liquid, and 4 = watery or with blood.

At days 4 and 8 PI, one pig per pen was euthanised for subsequently sampling during the morning (between 8:00 and 14:00). On day 4 PI, the intermediate initial BW and on day 8 PI, the heaviest initial BW animals were selected, respectively. A 10-mL blood sample was obtained by venepuncture of the cranial vena cava using 10-mL tubes without anticoagulant (Aquisel), and immediately animals received an intravenous injection of

sodium pentobarbital (200 mg/kg BW; Euthasol, Esteve). Once dead, animals were bled, the abdomen was immediately opened, and the intestinal tract excised.

Digesta samples from ileum and proximal colon (5 cm from caeco-colic junction) were collected, homogenised and immediately pH was determined with a pH-meter (Crison 52-32 electrode, Net Interlab), calibrated on each day of use. Different aliquots were sampled from homogenised digesta. Aliquots of approximately 2 g were kept on ice for microbiological analysis, being processed in less than 4 h. Samples of approximately 5 g were kept immediately on dry ice until being stored at -20 ºC for further analysis of short-chain fatty acids (SCFA) and lactic acid. Other similar aliquots were also kept immediately on dry ice previous to be stored at -80 ºC for DNA analysis for further *E. coli* F4 quantification by real-time PCR (quantitative PCR [qPCR]) and for microbiota high-throughput sequencing (HTS) (colon samples). A set of ileal and colonic digesta samples were preserved in a H_2SO_4 solution (3 mL of digesta plus 3 mL of 0.2 M H_2SO_4) and kept frozen at -20 ºC until ammonia (NH_3) determination.

To analyse the bacteria attached to the ileal mucus and epithelium, 15-cm long sections of ileum were excised, washed thoroughly (3x) with sterile PBS, opened longitudinally, and scraped with a microscope glass slide to obtain the samples. One aliquot was kept on ice and a second aliquot on dry ice for microbiological plate counts and pathogen quantification by qPCR respectively.

For histological study, 3-cm long sections from the distal ileum were removed, opened longitudinally, washed thoroughly with sterile PBS and fixed by immersion in a formaldehyde solution 4 % (Panreac).

Blood samples were centrifuged (1500 x *g* for 15 min) after refrigeration, and the serum obtained was divided in different aliquots and stored at -20 ºC.

Analytical procedure

Chemical analyses of the diets including dry matter (DM), ash, crude protein and diethyl ether extract were performed according to the Association of Official Agricultural

Chemists standard procedures (AOAC, 2007). Neutral-detergent fibre and acid-detergent fibre were determined according to the method of Van Soest et al. (1991).

Serum concentrations of tumour necrosis factor-α (TNF-α) was determined by the Quantikine Porcine TNF-α kit (R&D Systems); and pig-major acute-phase protein (Pig-MAP) concentration was determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A).

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned at 4-µm thickness and stained with haematoxylin and eosin. Morphological measurements of ten different villus-crypt pairs were performed with a light microscope (Leica DM5000B) (Jenoptik) fitted to a ProgRes® CapturePro software (Jenoptik) using the technique described in Nofrarías et al. (2006).

Ammonia (NH_3) concentration was determined with the aid of a gas-sensitive electrode (Hach Co.) combined with a digital voltmeter (Crison GLP 22, Crison Instruments S.A) following Barba-Vidal et al. (2017a). Briefly, three grams of digesta preserved in H_2SO_4 (1:1) were centrifuged at 1370 x *g* for 10 minutes. Supernatant was obtained and finally neutralised with 1 mL of 10 M NaOH to reach pH 11 while stirring and measuring the ammonia released as voltage (mV). Short-chain fatty acids (SCFA), and lactic acid were determined based on the method of Richardson et al. (1989) modified by Jensen et al. (1995) performed by gas chromatography after submitting the samples to an acid–base treatment followed by a diethyl ether extraction and derivatisation with *N*-(tertbutyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MBTSTFA) plus 1 % *tert*-butyldimethylchlorosilane (TBDMCS) agent.

For microbial counts of enterobacteria and total coliforms in digesta, faeces and ileal scrapings, samples were 10-fold serial diluted in Lactated Ringer's solution (Sigma-Aldrich) and seeded in MacConkey agar and chromogenic agar (Oxoid), respectively. Counts were read after 24 h incubation at 37 ºC.

E. coli F4 was quantified in colonic digesta and ileal scrapings by qPCR using a SYBR green dye. DNA from samples (approximately 250 mg) was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen), and subsequently its concentration and purity checked using NanoDrop 1000 Spectrophotometer (Thermo Fisher). All

recommended optimisation steps from the protocol were followed in order to improve bacterial cell rupture and purity. The DNA was finally stored at -80 ºC until use. A qPCR targeting the gene coding the F4 fimbria of ETEC F4 was performed by modifying the procedure described by Gustavo Hermes et al. (2013). Changes consisted in using a SYBR Green PCR Master Mix PCR kit (Applied Biosystems) and reducing the total volume of reaction to 20 µL, which included 10 µL of 2X SYBR Green PCR Master Mix PCR buffer, 0.88 µL of each primer (12.5 µM) and 5 µL of DNA sample.

In the case for HTS of colonic microbiota, the V3-V4 region of the 16S rRNA gene was targeted using the kit MiSeq® Reagent Kit v2 (500 cycle) (MiSeq from Illumina). Primers used in the construction of libraries with amplicons of putative 460 bp were the following:

F-5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

R-5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Sequence reads generated from MiSeq Illumina® system were processed on QIIME v.1.9.1 pipeline (Caporaso, 2010) with default settings. The quality filter of already demultiplexed sequences was performed at a maximum unacceptable Phred quality score of Q20. Resulting reads were clustered to operational taxonomic unit (OTUs) using uclust with a 97 % sequence similarity and picked by an open reference method (Rideout et al., 2014) at 10 % of sequences subsampled. Representative sequences were assigned to taxonomy against bacterial 16S GreenGenes v.13.8 reference database (DeSantis et al., 2006) at a 90 % confidence threshold and sequence alignment and phylogenetic tree building were obtained through uclust and FastTree. Chimeric sequences were removed with ChimeraSlayer (Haas et al., 2011), and further quality filtering consisted in removing singletons and OTUs with relative abundance across all samples below 0.005 % as recommended by Bokulich et al. (2013).

Statistical analysis

The effect of the experimental treatments on slaughter measurements was performed in free software R v3.4 using the stats package (R Core Team, 2013) lm() function for one-way ANOVA with diet as fixed effect. In the case of Pig-MAP variable, concentration levels

were analysed with likelihood.test() function from the same package for a frequency analysis as well as the number of animals with quantifiable levels of fermentation products followed the glm() function under a binomial distribution.

Average daily feed intake, and daily faecal consistency were performed using the lme4 package (Douglas et al., 2015) lmer() function for a linear mixed model (LMM) with a treatment-by-time interaction term.

The pen was used as the experimental unit. The alpha level for the determination of significance for all the analysis was 0.05. The statistical trend was also considered for *P* values >0.05 and <0.10. Data are presented and residual standard error (RSE).

For the biostatistics of quality-filtered sequences, firstly, OTU table was imported to R with phyloseq package (McMurdie & Holmes, 2013). Only OTUs shared among experimental groups were included and unique OTUs excluded. Alpha diversity (Chao1 and Shannon indexes) and beta diversity (Whittaker index) were calculated using vegan package (Oksanen, 2017) and tested for treatment effects with an ANOVA. For ordination analysis, the dissimilarity matrices based on Bray-curtis distances were estimated for each experimental group and were analysed with the non-linear multi-dimensional scaling (NMDS) using vegan package. The vegan envfit() function was used to determine if the ordinations differed between treatments within each trial, and also anosim() function was used for the analysis of similarities (ANOSIM). For further analysis of differences on taxa abundances between treatments, OTU and taxa relative abundances were used. The analysis consisted of a zero-altered negative binomial model with pscl package (Zeileis et al., 2008) or a negative binomial model with mass package (Venables & Ripley, 2002), and corrected by false-discovery rate (FDR) (Benjamini & Hochberg, 1995).

6.3 Results

Most of the animals showed a good health status at the beginning of the experiment and most of them adapted to facilities and feed positively. Nonetheless, several casualties took place on both trials, all from different pens. In Trial 1, one animal from CTR group

died before the challenge and two more casualties were found within both groups CTR and BUT+ on day 2 PI. In Trial 2, one animal in CTR group died on day 1 PI, and in HPT+ group, one casualty occurred before the challenge and other two on days 2 and 3 PI, respectively.

Performance

Initial BW were similar within trials. The effect of the experimental treatments on ADG, ADFI and GF are shown in Table 6.2. During the first week post-weaning (pre-inoculation), animals had an ADFI between around 100-200 g/day among trials that reached 200-260 g/day the day before the challenge. After the challenge, all animals reduced their feed intake (P_{day} <0.001) to an average between 100-170 g/day (data not shown). No differences were observed in performance parameters regarding treatments.

Table 6.2 Effect of experimental treatments on animal performance in Trial 1 and Trial 2.

	Trial 1				**Trial 2**			
	CTR	**BUT+**	**RSE**	***P***	**CTR**	**HPT+**	**RSE**	***P***
BW (kg)								
Initial	5.78	5.79	0.076	0.744	5.62	5.60	0.087	0.745
Final	8.30	7.67	0.995	0.222	7.77	7.51	0.786	0.592
ADFI (g/day)								
Pre-inoculation[A]	107	105	30.3	0.913	178	194	48.9	0.517
Post-inoculation[B]	255	211	56.5	0.137	259	254	54.5	0.865
ADG (g/day)								
Pre-inoculation[A]	52.5	31.5	52.0	0.434	77.4	86.0	41.8	0.686
Post-inoculation[B]	180	166	76.0	0.717	194	159	66.5	0.299
GF								
Pre-inoculation[A]	0.434	0.236	0.403	0.342	0.418	0.433	0.168	0.862
Post-inoculation[B]	0.661	0.788	0.268	0.358	0.725	0.633	0.185	0.339

[A] Pre-inoculation period included 0 – 7th day before inoculation. [B] Post-inoculation period included 0 – 8th day post-inoculation period. Legend: BW, body weight; ADFI, average feed intake; ADG, average daily gain; and GF, gain-to-feed ratio.

Clinical signs

A peak of diarrhoea was observed after both challenges being less severe in Trial 2 (from 1.21 ± 0.262 on day 0 PI to 1.58 ± 0.531 on day 1 PI; P_{day} = 0.08), showing animals from Trial 1 worsened faecal scores already before the challenge (from 1.69 ± 0.554 on day 0

PI to 2.30 ± 0.584 on day 1 PI; P_{day} <0.001). Treatments did not show differences on consistencies compared to CTR groups.

Inflammatory response

Serological concentrations of TNF-α and Pig-MAP are shown in Table 6.3. TNF-α was increased by BUT+ supplementation on day 8 PI (*P* = 0.023). In the case of Pig-MAP values, no significant impact of the treatments was detected.

Table 6.3 Effect of experimental treatments on serum inflammatory response on days 4 and 8 PI in Trial 1 and Trial 2.

	Trial 1				Trial 2			
	CTR	**BUT+**	**RSE**	***P***	**CTR**	**HPT+**	**RSE**	***P***
TNF-α (pg/mL)								
4 PI	101	135	54.0	0.231	156	124	43.4	0.159
8 PI	87.2	107	15.9	0.023	112	108	17.1	0.617
Pig-MAP (ranks[B])								
4 PI								
L-B-H	2-3-3	5-1-2	-	0.276	5-1-2	5-2-1	-	0.712
8 PI								
L-B-H	6-1-1	7-1-0	-	0.481	6-0-2	4-2-2	-	0.204

[A] Pig-MAP ranges as L-B-H: Low (0.3-1 mg/mL), borderline (1 – 2 mg/mL), and high (>2 mg/mL) levels. Legend: TNF- α, tumour necrosis factor-α; and Pig-MAP, pig major acute-phase protein.

Histomorphometry

On Table 6.4 are presented the changes produced by the diets in ileal histomorphometry. Despite no statistically differences, on day 8 PI BUT+ animals showed longer villi than CTR ones (*P* = 0.225) whereas HPT+ had shorter villi than CTR group (*P* = 0.155), accompanied by a lower villus:crypt ratio (*P* = 0.108) and previous shortened crypts on day 4 PI (*P* = 0.107). Goblet cells were also modified by HPT+ on day 4 PI with reduced numbers (*P* = 0.018) but increased with BUT+ on day 8 PI (*P* = 0.023). Neither IEL nor mitoses were changed with the additives.

Table 6.4 Effect of experimental treatments on ileal histomorphometry on days 4 and 8 PI in Trial 1 and Trial 2.

	Trial 1				Trial 2			
	CTR	**BUT+**	**RSE**	***P***	**CTR**	**HPT+**	**RSE**	***P***
Villus height (µm)								
4 PI	272	269	62.5	0.906	278	264	80.1	0.740
8 PI	305	350	70.5	0.225	323	266	75.7	0.155
Crypt depth (µm)								
4 PI	183	170	27.8	0.459	196	170	29.2	0.107
8 PI	186	178	26.3	0.595	189	199	24.6	0.425
Villus:Crypt ratio								
4 PI	1.54	1.64	0.457	0.669	1.47	1.65	0.516	0.518
8 PI	1.79	2.07	0.555	0.326	1.78	1.39	0.448	0.108
IEL (cells/100 µm)								
4 PI	2.09	1.88	0.485	0.405	2.07	2.19	0.498	0.634
8 PI	3.03	2.65	1.025	0.466	2.39	2.72	0.459	0.176
GC (cells/100 µm)								
4 PI	2.16	1.45	1.057	0.199	1.75	1.35	0.280	0.018
8 PI	0.96	1.63	0.524	0.023	1.31	1.65	0.669	0.326
MIT (cells/100 µm)								
4 PI	0.092	0.118	0.077	0.508	0.039	0.065	0.042	0.259
8 PI	0.095	0.117	0.077	0.560	0.052	0.030	0.036	0.250

Legend: IEL, intraepithelial lymphocytes in villi; GC, goblet cells in villi; and MIT, mitoses in crypts.

Fermentation

Table 6.5 shows the changes promoted by the experimental treatments on pH and the main ileal and colon fermentation products. In ileum, neither pH or ammonia amounts were modified by BUT+, however, animals receiving HPT+ diet presented a lower pH on day 8 PI ($P = 0.070$). Regarding fermentation products, lactic acid was the main compound followed by acetic acid. Ileal lactic acid concentration was not significantly modified by any of the treatments. Regarding acetic acid, it was not detected in all the animals, and HPT+ diet promoted a reduction ($P = 0.042$) in the number of animals with concentrations below the minimum detection levels (2.40 mM). No effects were registered for BUT+ diet.

In colon, HPT+ diet was associate with a reduction in pH at day 4 PI ($P = 0.025$) and no significant changes were detected with the diets in ammonia concentration. Regarding the concentration of SCFA, feeding the animals with BUT+ diet did not change the concentration of total SCFA but promoted changes in the molar proportion of some acids. BUT+ diet tended to increase the proportion of valeric acid at day 8 PI ($P = 0.068$) and the

relative abundance of BCFA (*P* = 0.105), as well as the absolute concentration (data not shown) (*P* = 0.052) at day 4 PI. Regarding the HPT+ diet, total SCFA (and absolute acetic, propionic and butyric acids alone) concentration was reduced on day 8 PI (*P* = 0.057) and the percentage of valeric acid was increased (*P* = 0.016).

Table 6.5 Effect of experimental treatments on ileum and colon fermentation on days 4 and 8 PI in Trial 1 and Trial 2.

	Trial 1				**Trial 2**			
	CTR	**BUT+**	**RSE**	***P***	**CTR**	**HPT+**	**RSE**	***P***
Ileum								
pH								
4 PI	6.88	6.80	0.332	0.653	6.81	6.71	0.220	0.395
8 PI	6.80	6.88	0.362	0.660	6.86	6.74	0.129	0.070
NH_3 (mM FM)								
4 PI	2.01	2.06	1.469	0.944	0.63	0.54	0.237	0.478
8 PI	1.58	1.23	0.629	0.285	0.98	0.80	0.329	0.270
Lactic acid (mM FM)								
4 PI	8.02	16.9	16.05	0.325	23.2	15.5	14.28	0.317
8 PI	23.2	31.7	30.26	0.625	19.1	23.6	19.36	0.658
Acetic acid[A] (mM FM)								
4 + 8 PI	7/13 (3.24)	6/14 (3.02)	-	0.568	13/15 (5.36)	8/15 (5.03)	-	0.042
Colon								
pH								
4 PI	6.41	6.35	0.636	0.859	6.26	6.01	0.190	0.025
8 PI	5.92	5.96	0.279	0.812	5.97	6.11	0.267	0.321
NH_3 (mM FM)								
4 PI	20.8	29.8	12.4	0.171	10.7	10.3	4.95	0.879
8 PI	22.4	23.5	7.48	0.781	16.3	16.3	4.21	0.976
Total SCFA (mM FM)								
4 PI	117	106	42.1	0.603	107	119	22.9	0.365
8 PI	131	139	23.7	0.511	155	132	21.5	0.057
Acetic acid[B] (%)								
4 PI	58.7	60.1	7.87	0.741	60.5	60.4	5.44	0.958
8 PI	58.8	57.0	5.47	0.515	61.3	62.1	4.51	0.738
Propionic acid[B] (%)								
4 PI	24.6	24.1	5.72	0.875	22.5	22.0	3.23	0.772
8 PI	24.5	24.6	4.77	0.952	22.6	22.0	2.46	0.620
Butyric acid[B] (%)								
4 PI	13.0	10.7	4.99	0.401	12.6	14.0	3.81	0.492
8 PI	13.3	13.6	3.33	0.870	13.5	12.2	3.19	0.420
Valeric acid[B] (%)								
4 PI	2.26	2.83	1.855	0.566	2.72	2.41	0.873	0.504
8 PI	2.13	3.28	1.162	0.068	1.59	2.56	0.705	0.016
BCFA[B] (%)								
4 PI	1.46	2.23	0.858	0.105	1.67	1.23	0.609	0.188
8 PI	1.24	1.49	0.504	0.326	0.95	1.16	0.386	0.292

[A] Proportion of animals with quantifiable amounts of acetic acid and concentration in brackets. [B] Each SCFA molar proportion relative to total SCFA. Legend: NH_3, ammonia concentration; SCFA, short-chain fatty acids; and BCFA, branched-chain fatty acids.

Microbiological plate counts

Regarding microbial counts of faecal samples on the day of arrival, we did not found differences between the experimental groups in any of the trials (9.03 ± 0.536 log CFU/g fresh matter (FM) and 9.76 ± 0.300 log CFU/g FM enterobacteria for Trial 1 and 2, respectively; 8.30 ± 0.206 log CFU/g FM and 9.67 ± 0.399 log CFU/g FM coliforms for Trial 1 and 2, respectively). However, after one week of adaptation, whereas BUT+ did not modify faecal microbial counts (9.46 ± 0.532 vs. 9.05 ± 0.943 enterobacteria log CFU/g FM, and 8.60 ± 1.239 vs. 8.72 ± 1.049 coliform log CFU/g FM for BUT+ and CTR, respectively; P >0.30), HPT+ tended to reduce coliform numbers (9.07 ± 2.45 vs. 10.9 ± 1.46 log CFU/g FM for HPT+ and CTR, respectively; P = 0.096) and numerically enterobacteria (10.3 ± 2.20 vs. 11.2 ± 1.64 log CFU/g FM for HPT+ and CTR respectively; P = 0.386).

Table 6.6 presents the microbiological analysis on days 4 and 8 PI in ileum and colon digesta and in ileal mucosa scrapes. In no one of the trials, treatments altered the bacteria attached in ileal mucosa. Within BUT+ group, animals presented at day 8 PI higher enterobacteria counts in ileal digesta by a mean +1.1 log CFU/g FM (P <0.001) and numerically higher in colonic digesta by a mean +0.64 log CFU/g FM (P = 0.193). With HPT+ diet, on day 4 PI enterobacteria decreased numerically in ileum by a mean -0.47 log CFU/g FM (P = 0.123) and in colon by a mean -0.85 log CFU/g FM (P = 0.156) compared to CTR.

In Table 6.7 is presented the prevalence of *E. coli* F4 determined by qPCR in ileal mucosa and colon digesta. Regarding the effect of BUT+, at day 4 PI, it was found a higher number of animals with detectable levels of *E. coli* F4 attached to ileal mucosa (100 %) compared to the CTR group (50 %; P = 0.009). At day 8 PI, *E. coli* F4 was detected in less than 20 % of the animals and differences due to treatment disappeared. No differences were found with BUT+ supplementation in colonic digesta. Regarding HPT+ diets, in the Trial 2 it was not possible to detect the pathogen in any of the ileal scraping samples (except one CTR animal at day 4 PI). In colonic digesta, the HPT+ diet promoted a decrease in the number of animals with quantifiable numbers of the pathogen at day 4 PI (P = 0.035). At day 8 PI, the pathogen was only quantifiable in 25 % of the animals without differences between diets.

Table 6.6 Effect of experimental treatments on microbial plate counts on days 4 and 8 PI in Trial 1 and Trial 2.

	Trial 1				Trial 2			
	CTR	BUT+	RSE	*P*	CTR	HPT+	RSE	*P*
Ileal mucosa								
Enterobacteria[A]								
4 PI	8.75	8.90	0.836	0.718	5.94	6.22	0.841	0.504
8 PI	7.17	7.54	0.880	0.411	5.83	6.15	1.232	0.604
Coliforms[B]								
4 PI	8.66	8.81	0.860	0.738	5.53	5.68	1.136	0.791
8 PI	6.99	7.06	0.772	0.868	5.20	5.52	1.174	0.599
Ileal digesta								
Enterobacteria[A]								
4 PI	7.70	8.29	1.544	0.457	6.81	6.34	0.548	0.123
8 PI	7.15	8.25	0.464	<0.001	6.62	6.31	1.171	0.600
Coliforms[B]								
4 PI	7.67	7.88	1.601	0.791	6.02	5.84	0.844	0.692
8 PI	7.34	7.74	0.720	0.292	6.42	6.13	1.157	0.621
Colonic digesta								
Enterobacteria[A]								
4 PI	9.57	9.51	1.034	0.902	10.70	9.85	1.083	0.156
8 PI	8.30	8.94	0.943	0.193	11.00	10.99	1.083	0.984
Coliforms[B]								
4 PI	9.53	9.15	1.125	0.509	8.92	8.56	0.946	0.477
8 PI	8.23	8.77	0.982	0.288	10.97	10.93	1.090	0.948

[A] Enterobacteria plate counts (log CFU/g FM). [B] Total coliform plate counts (log CFU/g FM).

Table 6.7 Effect of experimental treatments on pathogenic *E. coli* F4 on days 4 and 8 PI in Trial 1 and Trial 2.

	Trial 1			Trial 2		
	CTR	BUT+	*P*	CTR	HPT+	*P*
Mucosa						
E. coli F4[A]						
4 PI	4/8 (6.88)	8/8 (5.99)	0.009	1/8 (8.13)	0/8	-
8 PI	1/8 (5.15)	2/8 (5.34)	0.519	0/8	0/8	-
Colon						
E. coli F4[A]						
4 PI	8/8 (6.35)	8/8 (6.56)	1.000	7/7 (5.13)	5/8 (5.25)	0.035
8 PI	5/8 (5.18)	7/8 (5.46)	0.240	2/8 (5.11)	2/8 (5.56)	1.000

[A] Log F4 gene copies/g FM. Detection limit of the method was 3.83-log F4 gene copy numbers/g FM. In brackets are shown the mean copy numbers for those animals with detectable levels (log F4 gene copies/g FM).

Microbiological 16S rRNA gene sequencing

To analyse global changes in the colonic bacterial populations by HTS, we selected the day 4 PI considering that most of the effects previously shown were observed at this day.

A total mean of 70,247 ± 52,504 number of reads/sample were obtained for Trial 1, and a mean 124,361 ± 104,303 number of reads/sample for Trial 2, with no differences among diets in any of the them (P >0.05). The analysis below was based on the OTU core, i.e. OTUs shared within each experimental groups pair (treated-CTR) within each trial. In this way, the OTU core included 769 and 806 OTU in Trial 1 and 2, respectively.

Richness was not affected by BUT+ (P = 0.317) nor HPT+ (P = 0.329), although four samples (tagged as 2, 10, 12, and 21) in the Trial 1 (half CTR and half BUT+) exhibited considerably lower number of OTU compared to the rest, as displayed in the rarefaction curves from Figure 6.1. Interestingly, these animals also were those with the highest amounts of *E. coli* F4 attached to the mucosa (8.5 ± 0.40 log F4 gene copies/g FM) compared to the rest of animals showing quantifiable levels (5.2 ± 1.17 log F4 gene copies/g; see Table 6.7). Also, compared to the rest, the same animals showed high *E. coli* F4 numbers in colon digesta (8.5 ± 2.05 vs. 5.78 ± 1.28 log F4 gene copies/g FM) and the lowest ADG from day 0 to 4 PI (-280 ± 285 vs. 185 ± 126 g/day).

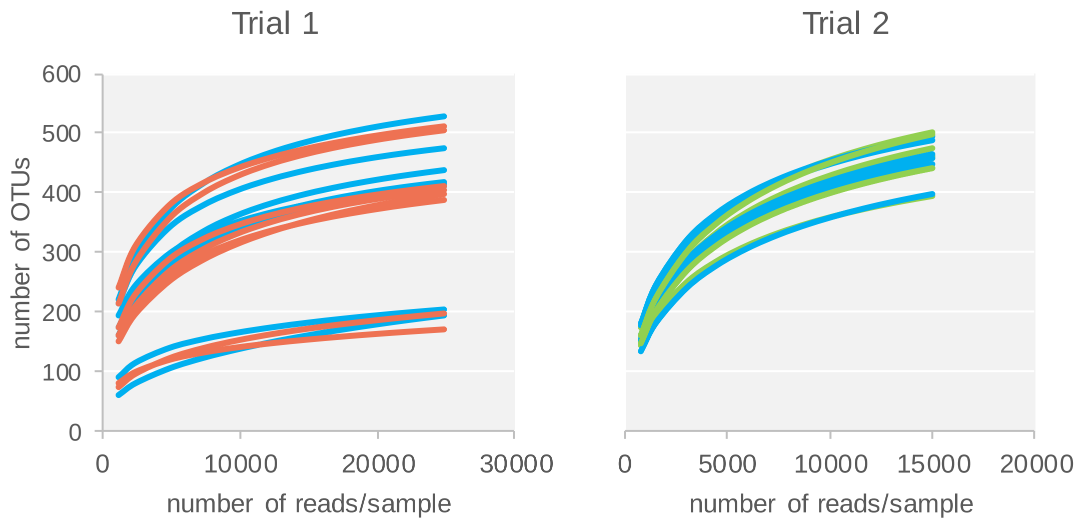


Figure 6.1 Individual rarefaction curves (calculated richness) from day 4 PI corresponding to CTR animals (-) and animals treated with BUT+ (-) in Trial 1 or treated with HPT+ (-) in Trial 2.

Regarding diversity, no differences between diets were found in Trial 1 nor Trial 2 in Shannon index (*P* = 0.846 and *P* = 0.967, respectively) nor Whittaker index (*P* = 0.874 and *P* = 0.756, respectively). The NMDS ordination did not differentiate clusters (data not shown) in any of the two trials (*P* >0.05), although the four samples with lowest richness from Trial 1 were separated from the rest as can be observed in the dendrogram of Figure 6.2.

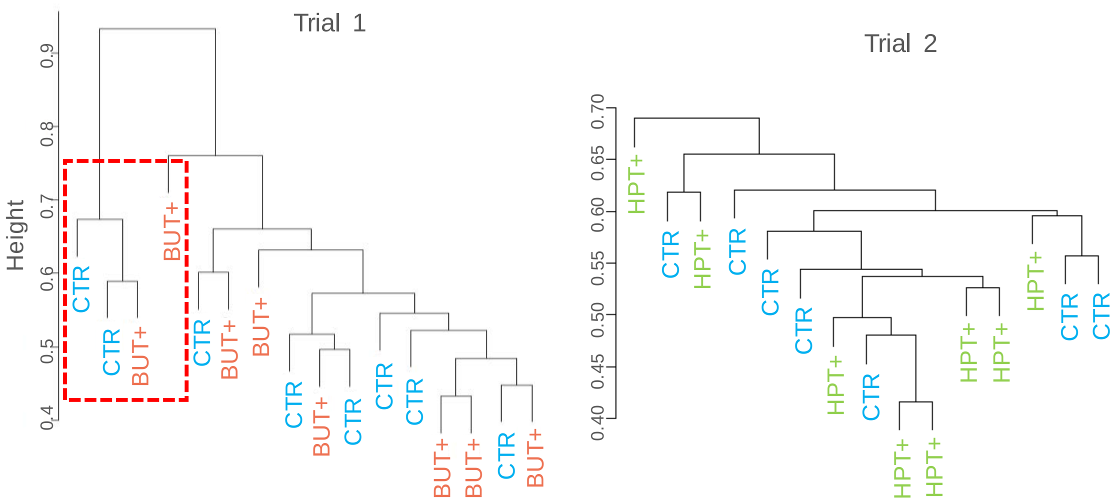


Figure 6.2 Dendrogram of UPGMA hierarchical clustering (average method) based on Bray-curtis distances from day 4 PI representing individual animals treated CTR diet or supplemented with BUT+ in Trial 1 or with HPT+ in Trial 2. Animals with putative dysbiosis (2, 10, 21, and 12) are outlined with a dashed-line box.

Regarding taxonomic groups, the major phyla were similarly represented in both trials as can be seen in Figure 6.3. Considering the whole of the analysed samples, Firmicutes represented around a 45 % and Bacteroidetes around a 40 %, followed by Proteobacteria around a 9 %. Regarding the effect of the diets on abundance of main phyla, no significant changes were detected in any of the trials. Despite this, Firmicutes:Bacteroidetes (F/B) ratio was increased with BUT+ (1.44 vs. 0.94 for CTR and BUT+, respectively; *P* = 0.054) but not modified by HPT+ diet (1.18 vs. 1.22 for HPT+ and CTR, respectively; *P* = 0.833).

In Figure 6.3 are also represented those four animals from Trial 1 that showed an unusual low richness (low number of OTUs) and could be considered as animals with a putative dysbiosis. Three of these animals (2, 10, 21) exhibited dramatic increases in the proportions of Proteobacteria plus Fusobacteria (higher than 50 %), at the expense of Firmicutes when compared to the rest of animals. At lower taxonomic levels, they showed higher proportions of some families that normally represent no more than 1 % of total population such as Enterobacteriaceae (17.4 %), Fusobacteriaceae (27.5 %),

Campylobacteraceae (16.9 %), Bacteroidaceae (9.70 %), and Pasteurellaceae (3.77 %). These increases were at the expense of other major ones, Prevotellaceae, Ruminococcaceae, and Lachnospiraceae.

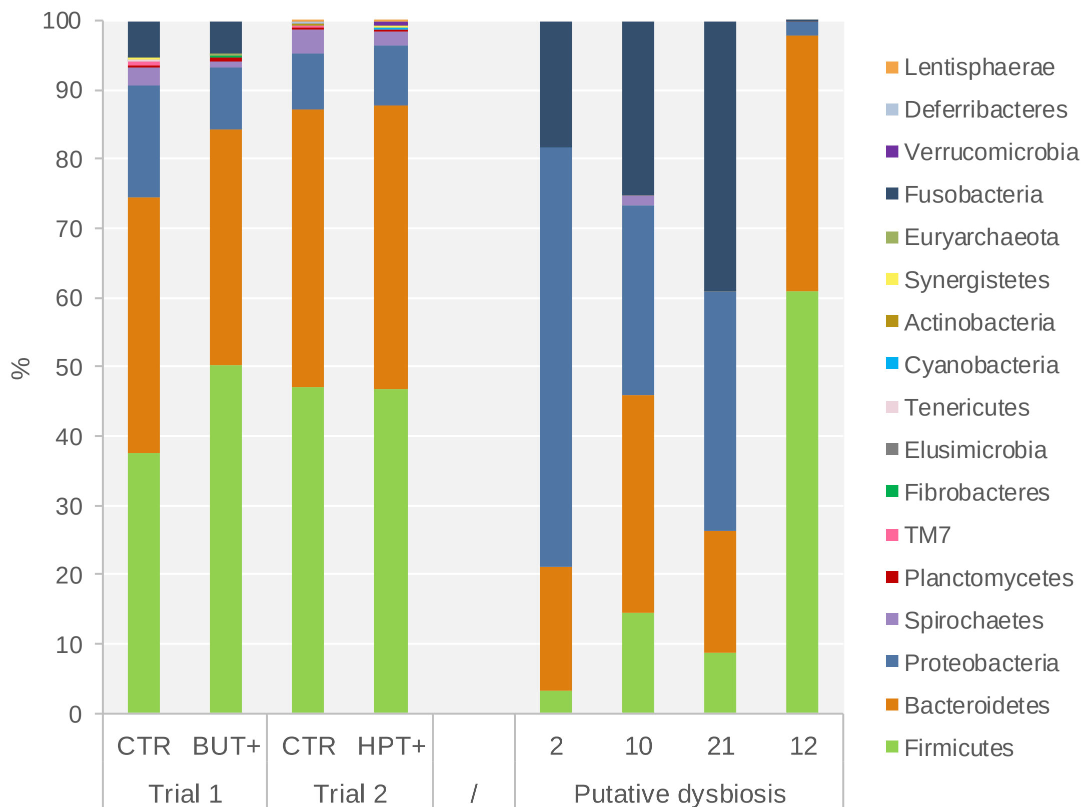


Figure 6.3 Relative abundances (%) of phyla represented as the average for CTR groups and animals treated with BUT+ in Trial 1 or treated with HPT+ in Trial 2 on day 4 PI. Animals with putative dysbiosis (2, 10, 21, and 12) are shown separately.

When evaluating the effect of the dietary treatments on abundances at family and genus level (Table 6.8), it can be seen that BUT+ diet reduced the number of candidates Paraprevotellaceae (4.98 ± 3.64 in BUT+ vs. 8.17 ± 4.62 % in CTR group) and *Prevotella* (6.39 ± 4.337 in BUT+ vs. 3.50 ± 2.357 % in CTR group) as well as *Phascolarctobacterium* (5.17 ±3.40 in BUT+ vs. 9.30 ± 3.57 % in CTR group). Both additives incremented *Lachnobacterium* compared to their respective CTR groups (0.261 ± 0.576 in BUT+ vs. 0.010 ± 0.016 % in CTR group, and 0.283 ± 0.675 in HPT+ vs. 0.002 ± 0.002 % in CTR group). This is a minor genus (<1 %) as well as the other groups modified by HPT+. These consisted of WCHB1-25 (0.021 ± 0.037 in HPT+ vs. 0.001 ± 0.001 % in CTR group), Pasteurellaceae (0.985 ± 1.721 in HPT+ vs. 0.100 ± 0.125 % in CTR group) and

Aggregatibacter (0.035 ± 0.067 in HPT+ vs. 0.002 ± 0.002 % in CTR group) that increased with HPT+. On the contrary, Peptococcaceae (0.011 ± 0.006 in HPT+ vs. 0.013 ± 0.019 % in CTR group) and Enterobacteriaceae (0.026 ± 0.042 in HPT+ vs. 0.243 ± 0.385 % in CTR group) were reduced with HPT+.

Table 6.8 Effect of experimental treatments on the relative abundance of families and genera on day 4 PI in Trial 1 and Trial 2.

	Taxonomic level	Taxon	Ln change	adjusted *P*
Trial 1	Family	cand. Paraprevotellaceae	-0.632	0.000
BUT+ vs. CTR	Genus	cand. *Prevotella*	-0.800	0.000
		Phascolarctobacterium	-0.072	0.000
		Lachnobacterium	3.987	0.073
Trial 2	Family	WCHB1-25	5.524	0.000
HPT+ vs. CTR		Peptococcaceae	-1.047	0.012
		Enterobacteriaceae	-2.309	0.027
		Pasteurellaceae	2.282	0.069
		Turicibacteraceae	-1.657	0.069
	Genus	*Lachnobacterium*	4.990	0.000
		Aggregatibacter	3.401	0.042

Positive values and negative values indicate greater and lower abundance, respectively, in treated animals (BUT+ or HPT+). Taxa are sorted by level of significance (from higher to lower; FDR-adjusted P <0.10). Only taxa detected in at least half of the samples are represented.

As changes in minor taxa are most likely linked to changes in specific OTUs, in Figure 6.4 are exhibited the OTUs for which significant differences were observed between treatments in each trial.

Trial 1

CTR	BUT+	OTU code		Taxonomic classification
0.724	0.003	856253	↓	Spirochaetes, Spirochaetales, Spirochaetaceae, *Treponema*
0.072	0.642	333426	↑	Firmicutes, Clostridiales
0.056	0.643	571178	↑	Firmicutes, Clostridiales, Lachnospiraceae, *Roseburia*
0.002	0.258	584463	↑	Firmicutes, Clostridiales, Lachnospiraceae, *Lachnobacterium*
0.225	0.031	28970	↓	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella*
0.035	0.193	780650	↑	Firmicutes, Clostridiales, Clostridiaceae
0.028	0.162	712142	↑	Firmicutes, Clostridiales, Ruminococcaceae
0.115	0.006	523621	↓	Cyanobacteria, YS2
0.117	0.004	99294	↓	Spirochaetes, Sphaerochaetales, Sphaerochaetaceae, *Sphaerochaeta*
0.009	0.107	522591	↑	Bacteroidetes, Bacteroidales, S24-7
0.029	0.073	545061	↑	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella, copri*
0.002	0.065	341730	↑	Firmicutes, Clostridiales, Lachnospiraceae, *Coprococcus*
0.008	0.035	363400	↑	Firmicutes, Clostridiales, Lachnospiraceae
0.027	0.009	303112	↓	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella*
0.018	0.006	New ref. 4408	↓	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella*
0.017	0.005	New ref. 2158	↓	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella copri*
0.003	0.014	619919	↑	Firmicutes, Clostridiales
0.002	0.016	370183	↑	Firmicutes, Clostridiales, Lachnospiraceae, *Blautia*
0.008	0.001	New ref. 3722	↓	Firmicutes, Clostridiales

Trial 2

CTR	HPT+	OTU code		Taxonomic classification
0.044	2.714	345899	↑	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella copri*
0.870	1.577	817140	↑	Firmicutes, Clostridiales, Veillonellaceae, *Megasphaera*
0.738	1.152	567226	↑	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella*
0.092	1.283	11235	↑	Proteobacteria, Campylobacterales, Campylobacteraceae, *Campylobacter*
0.148	1.096	813654	↑	Firmicutes, Clostridiales, Lachnospiraceae
0.293	0.055	924224	↓	Spirochaetes, Spirochaetales, Spirochaetaceae, *Treponema*
0.073	0.236	338992	↑	Firmicutes, Clostridiales, Lachnospiraceae
0.004	0.305	New ref. 7582	↑	Bacteroidetes, Bacteroidales, cand. Paraprevotellaceae, cand. *Prevotella*
0.002	0.282	584463	↑	Firmicutes, Clostridiales, Lachnospiraceae, *Lachnobacterium*
0.218	0.026	New ref. 5389	↓	Firmicutes, Clostridiales, Lachnospiraceae
0.220	0.017	782953	↓	Proteobacteria, Enterobacteriales, Enterobacteriaceae
0.002	0.128	339221	↑	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella copri*
0.003	0.101	348703	↑	Firmicutes, Clostridiales, Lachnospiraceae, *Coprococcus*
0.006	0.090	733799	↑	Bacteroidetes, Bacteroidales
0.010	0.082	New ref. 4778	↑	Firmicutes, Clostridiales, Lachnospiraceae
0.003	0.061	1027100	↑	Bacteroidetes, Bacteroidales
0.006	0.053	471412	↑	Proteobacteria, Pasteurellales, Pasteurellaceae
0.045	0.009	752354	↓	Firmicutes, Clostridiales, Lachnospiraceae, *Coprococcus*
0.052	0.001	New ref. 6830	↓	Firmicutes, Clostridiales
0.038	0.009	362342	↓	Firmicutes, Clostridiales, Ruminococcaceae, *Ruminococcus*
0.034	0.008	321484	↓	Firmicutes, Clostridiales, Ruminococcaceae, *Oscillospira*
0.002	0.035	9498	↑	Proteobacteria, Pasteurellales, Pasteurellaceae, *Aggregatibacter*
0.004	0.030	350627	↑	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella copri*
0.023	0.003	4296764	↓	Firmicutes, Clostridiales, Christensenellaceae
0.020	0.003	510777	↓	Firmicutes, Clostridiales, Lachnospiraceae
0.004	0.017	New ref. 2007	↑	Bacteroidetes, Bacteroidales, cand. Paraprevotellaceae, CF231
0.001	0.021	295878	↑	Verrucomicrobia, WCHB1-41, WCHB1-25
0.004	0.014	New ref. 2891	↑	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella copri*
0.008	0.010	527941	↑	Bacteroidetes, Bacteroidales, Prevotellaceae, *Prevotella copri*
0.011	0.004	540862	↓	Firmicutes, Clostridiales, Ruminococcaceae
0.010	0.001	New ref. 5638	↓	Firmicutes, Clostridiales, Lachnospiraceae

Figure 6.4 Heatmap of the effects of experimental additives BUT+ in Trial 1, and HPT+ in Trial 2 on the relative abundance of OTUs (%) change against CTR (adjusted-FDR *P* value <0.05). Arrows indicate if the OTU increased (↑) or decreased (↓) in treated animals compared to CTR. Relative abundance from 0 % (yellow) to 3 % (green). Only OTUs present in at least half of the animals per experimental group were evaluated.

With BUT+, most of the changes consisted of increases of OTUs of the Clostridiales order (nine OTUs), in accordance to the higher F/B exposed before. These included OTUs classified as *Roseburia*, *Lachnobacterium*, *Coprococcus* and *Blautia* genera, as well as two within the Clostridiaceae (OTU-780650) and Ruminococcaceae (OTU-712142) families. On the contrary, OTUs belonging to *Treponema* (OTU-856253), the cyanobacterium YS2 (OTU-523621), *Spaerochaeta* (OTU-99294) and *Prevotella* (four OTUs) genera were lower with BUT+ as reported in the taxon differential test of Figure 6.4. Regarding HPT+ diet (Trial 2), and accordingly to Table 6.8, most of the OTUs that were significantly reduced by the additive (9/11) were classified within Clostridiales order under the Lachnospiraceae (four OTUs), Ruminococcaceae (three OTUs) and Christensenellaceae (OTU-4296764) families. Other OTUs of Clostridiales order classified as Lachnospiraceae (4 OTU) and Veillonellaceae (*Megasphaera*, OTU-817140) families were, however, enhanced. Up to 10 OTUs classified within the Bacteroidetes phylum were increased by HPT+, most of them (6 OTUs) assigned to *Prevotella* genus and two (new ref. OTUs 7582 and 2007) to the candidate family Paraprevotellaceae. Four of the modified OTUs belonged to the Proteobacteria phylum, being three of them increased by HPT+ corresponding to *Campylobacter* (OTU-11235), *Aggregibacter* genus (OTU-9498) or Pasteurellaceae family (OTU-471412), and one decreased belonging to the Enterobacteriaceae family (OTU-782953). Other minor groups were also modified as shown in Figure 6.4.

6.4 Discussion

The rise of antibiotic resistances needs to be solved into a multidisciplinary approach (OiE, 2016), being one of the strategies finding in-feed alternatives. With this purpose, in previous studies of our group we tested two forms of fat-protected sodium butyrate or sodium heptanoate in front of an oral ETEC F4 challenge in weaned piglets. In that work, protected forms of sodium butyrate and sodium heptanoate were demonstrated to be able to modify the fermentative patterns along the whole GIT and modify several microbial groups at colon like *Prevotella* or minor populations from Clostridiales and Bacteroidales (Chapter 4). In a second stage of research, we also tested a blend of sodium

salts of fatty acids derived from coconut distillates consisting of MCFA, mainly lauric acid (C12), under oral challenges with ETEC F4 or *Salmonella* Typhimurium. In this case, the MCFA mixture showed reductions on the pathogen loads at the distal part of the gut (Chapter 5) and also changes in some microbial groups depending on the pathogen challenge. Increases were detected on the Fibrobacteraceae family under the *Salmonella* challenge and after the ETEC F4 challenge there were found increases in the Veillonellaceae family and related members. Considering these previous results, in the present work, the two sodium salts, butyrate and heptanoate, were protected with the sodium salt of a MCFA blend looking for synergistic effects and providing a potential antibiotic replacer.

For this aim, we performed two independent trials to test each one of the additives against a CTR diet. The two challenges triggered slightly different outcomes. In Trial 1, an obvious peak of diarrhoea was observed, and the inflammatory biomarkers used were clearly boosted in several animals exhibiting values beyond the normal range (ca. >100 pg/mL for TNF-α, and ca. >1 mg/mL for Pig-MAP) (Piñeiro et al., 2009; Barba-Vidal et al., 2017b). In the Trial 2, the course of diarrhoea was milder, and animals showed lower loads of ileal enterobacteria and coliforms. However, despite differences in clinical outcomes in both trials, animals displayed similar average levels of pro-inflammatory biomarkers and the pathogen *E. coli* F4 could be detected in both trials at the colonic digesta.

No significant effects were registered in performance parameters with the use of the experimental diets. In this regard, it should be beard in mind that the objective of this work, rather than evaluating the effects on performance, was to study the clinical and global gut health response of the weaned piglet after the oral challenge. This implies the use of experimental models of disease with a reduced number of animals that limits the capability of the design to detect effects on performance. In that sense, previous works evaluating fat-protected sodium butyrate or heptanoate (Chapter 4) or the blend of sodium salts of MCFA from coconut distillates (Chapter 5) were neither able to detect significant effects on animal performance. Other authors have, however, registered improvements in feed efficiency when higher levels of MCFA are used, associated to the provision of a rapid energy source to the animal (Geng et al., 2016a), or also

improvements in feed consumption and growth with the use of different combinations of short- and medium-chain fatty acids (Kuang et al., 2015; Lei et al., 2017; Li et al., 2018). However, these works are hardly comparable since they evaluated different active principles, different doses and under health status conditions.

Effects of BUT+ (sodium butyrate protected with coconut distilled MCFA sodium salts)

As stated in the introduction, SCFA and MCFA as in-feed additives are proposed as a mean to improve gut health and prevent post-weaning diarrhoea by exerting synergistic effects (Vande Maele et al., 2016). Regarding the potential of BUT+ to reduce the ability of the pathogen to colonise the gut, results from our study do not seem to confirm this hypothesis. Whereas only half of the animals from the control group showed detectable levels of *E. coli* F4 attached to ileal mucosa at day 4 PI, all the animals receiving BUT+ presented the pathogen. Consistently with these results, higher plate counts of enterobacteria were also found in ileum digesta at day 8 PI in BUT+ animals. This apparent higher colonisation of the small intestine by enterobacteria and *E. coli* F4 could be related to the higher values found in serum TNF-α at day 8 PI with BUT+ (P = 0.023). Moreover, the higher colonic concentrations of BCFA, and valeric acid with BUT+ could be also related to the high proteolytic activity described for the enterobacteria group (Gilbert et al., 2018). Regarding the possible promoting effect of BUT+ on enterobacteria, previous results of our group also found increased numbers of ileal enterobacteria with sodium butyrate. Also, higher colonic concentrations of BCFA and valeric acid were found when the MCFA mixture was given alone (Chapter 5). Nonetheless, it is generally accepted that butyrate nourishes the intestinal epithelia and also can avoid the translocation of pathogens under stressing conditions (Lewis et al., 2010). However, despite the increase in the number of enterobacteria attached to ileum and *E. coli* F4, the ileal villi from the animals fed BUT+ were not injured but even numerically longer (P = 0.225) compared to CTR. The role of butyrate as an energy source for enterocytes (Long et al., 2018) could had been counteracting detrimental effects of *E. coli* on them, and moreover, the increase in the mucin synthesis that some authors have reported for sodium butyrate (Gaudier et al., 2004) could have aided to limit the invasiveness of the pathogen, considering the higher number of goblet cells found at day 8 PI with BUT+.

When evaluating the effect of SCFA and/or MCFA on the microbiota, most of the studies observed a modulatory rather than an antimicrobial activity on gut bacteria. Long et al. (2018) were able to see reductions of *E. coli* with a blend of SCFA (formic, acetic and propionic) and MCFA, and Kuang et al. (2015) increases of lactic acid bacteria (LAB) with a mixture of formate, lactate and citric acid with MCFA in faeces from weaned piglets. Other authors (Zentek et al., 2013; De Smet et al., 2016) found variations in other bacterial groups only on the upper parts of the GIT. Furthermore, the results from other studies analysing the impact of these blends on the microbiota through the 16S rRNA sequencing (Li et al., 2018; Soler et al., 2018) reinforce the idea that, at least in colon, no generalised antimicrobial action exists since the richness (number of OTUs) and the sequencing depth were not modified. In our work, neither we were able to detect structural changes in the microbiota ecosystem related to the experimental diets, nor in richness nor in biodiversity. Only in four animals from the first trial, being those with the highest amounts of *E. coli* F4 attached to the mucosa, we were able to detect dramatic changes in the microbiota ecosystem not related to the experimental diets. These four animals probably were facing a clear dysbiosis due to the oral challenge with the predominance of Proteobacteria and Fusobacteria in colonic contents at the expense of Firmicutes. Up to our knowledge, very few works have assessed the impact of an oral challenge in the microbiota of weaned piglets. Only the work of Argüello et al. (2018) showed the immediate changes promoted after a *Salmonella* Typhimurium challenge by 16S rRNA sequencing. Other published studies with chicken (Mon et al., 2015; Li et al., 2017) or human enteric pathogens (Schneider et al., 2017; Burdet et al., 2018; Dong et al., 2018) have also described structural changes in microbiota. Nonetheless, despite the inherent differences between species, a clear idea was determined, that in the dysbiotic ecosystem a clear differentiated microbial structure was driven by the proliferation of Proteobacteria groups detrimental to those from Firmicutes or Bacteroidetes.

Despite BUT+ did not promote big structural changes in the colonic ecosystem, we were able to detect changes in some particular groups that would suggest ability of this additive to modulate colonic microbiota. The most remarkable shift observed was the increase of the ratio Firmicutes:Bacteroidetes (F/B) that could be regarded as positive. When sodium butyrate was tested alone in previous studies, the F/B ratio, however,

tended to be reduced (Chapter 4) and when the sodium salt of MCFA blend from coconut distillates was tested, we found a numerical increase in the F/B ratio although not significant (Chapter 5). These results would suggest that sodium butyrate protected by sodium salt of MCFA in this study could had driven the changes observed in colonic bacterial populations and their fermentative activity. Actually, valeric acid production was also increased when the coconut distillate was tested alone. From this point of view, the role of butyrate in this synergic blend could have been more related to maintaining the barrier integrity and no so much modulating the colonic microbiota. Nonetheless, these hypotheses have to be read with caution considering that changes in microbiota were assessed at different tempos, since were marked, and thus measured, at different days (butyrate alone or protected with coconut distillates on day 4 PI, and coconut distillates alone on day 8 PI). Furthermore, these effects include the differences on coliform plate counts evaluated for butyrate alone (increased at day 4 PI) and MCFA coconut distillates (decreased at day 8 PI) in ileum.

In here, exploring the drift observed on F/B ratio, on one hand, there was the reduction of *Phascolarctobacterium* genus, within Bacteroidetes, and on the other hand, shifts seen at OTU level related to the increases of members from Ruminococaceae, Lachnospiraceae and Clostridiaceae families, concomitant with the decreases from the candidate *Prevotella* and members from *Prevotella* genus. Two enterotypes have been described in the literature with healthy pigs (Mach et al., 2015; Ramayo-Caldas et al., 2016), resembling those previously described for humans (Arumugam et al., 2011). These two enterotypes include *Prevotella* and Ruminococcaceae as main representatives, the latter accompanied with close relatives such as Lachnospiraceae (Arumugam et al., 2011). Actually, some OTUs classified within this group were significantly promoted in animals supplemented with BUT+ belonging to the genera *Blautia*, *Lachnobacterium*, and *Roseburia*. Precisely, the Ruminococcaceae driven enterotype previously described has been associated to a (pre-weaning) lactating stage due to the metabolic profile of the bacteria, including overrepresentation of *Escherichia* (Mach et al., 2015). Moreover, Ramayo-Caldas et al. (2016) associated this enterotype with a complex cross-feeding system involving butyrate, nitrogen and amino acids, although it must be considered that the analysis was based on fattening animals. With this regard, it could be hypothesised

that animals treated with BUT+ might present a developing microbial gut ecosystem with still higher presence of populations typical from pre-weaning (Petri et al., 2010) such the increase of enterobacteria could have been a consequence of a transient microbiota and gut ecosystem adapting to stress, in our case the ETEC F4 challenge.

Effects of HPT+ (sodium heptanoate protected with coconut distilled MCFA sodium salts)

Contrary to BUT+, HPT+ was shown to be effective limiting colon colonisation by *E. coli* F4 (day 4 PI, $P < 0.05$) with consistent numerical reductions of enterobacteria and coliforms in ileum and colon compared to the control group ($P < 0.20$). These reductions could be related to the fact that HPT+ had been capable of reducing the faecal coliforms even before the challenge. A reduction in the colonisation of the gut by the pathogen at day 4 PI could be related to the numerical reductions observed in the TNF-α serum levels ($P = 0.159$) and also the trend for shorter crypt depths ($P = 0.107$) at day 4 PI. The reduced counts of goblets cells observed in ileum would have triggered a different casuistic to that discussed with BUT+. This decrease could be due to the reduced need to produce mucin as a protective strategy, if not induced by the HPT+ diet, reducing possibilities of ETEC F4 to adhere to the epithelium.

Together to the reductions observed in pathogen colonisation with HPT+, it was also found a diminution in fermentation products along the gut. Acetic acid presence in ileum was reduced, as well as colonic total SCFA concentration (acetic, propionic and butyric acids) at day 8 PI ($P = 0.057$). Similar effects were found in the previous works with coconut distillates alone (Chapter 5), where also there were found increases in the molar percentage of valeric acid. These results are in line with the work of Zentek et al. (2013) where combining classical short-chain organic acids (SCOA) (1.05 % of fumaric and lactic acids) with MCFA (0.3 % of caprylic and capric acids) also depressed the intestinal fermentation. These changes might suggest that the supplemented combination could have aided in the digestion in the upper parts as has been observed in other works (Hanczakowska et al., 2011), thus limiting the available substrate reaching the hindgut.

These changes on the fermentation profile suggest possible changes induced by the additive in the colonic microbiota that in turn could have had a feed-back with the host.

However, when analysed by HTS, this decreased SCFA were not accompanied by a lower presence of total microbes with HPT+, what could not lead to affirm an antibacterial effect. Another explanation could have been that both heptanoate and MCFA coconut distillates could have sum up and impact in a different way to that observed with BUT+. In this case, the most notable effect was the reduction of the percentage of Enterobacteriaceae consistent with the reductions on plate counts. It is true that in the previous work with MCFA (Chapter 5), it was suggested that the decrease of commensal bacteria, such are enterobacteria, might had stimulated the local immunity (intraepithelial lymphocytes) since it could be associated to a harmful signal, but in that case *E. coli* F4 charges were not modified as occurred in the present experiment. Precisely, the addition of MCFA to sodium heptanoate could have allowed the possibility to exert in the distal parts the inhibitory effects seen on stomach by sodium heptanoate alone (Chapter 4), detrimental to other populations and accompanied by a decreased pH. In this sense, despite the reduction of the pathogenic ETEC, other potential pathogens such those belonging to Pasteurellaceae were enhanced by HPT+, possibly related to a restitution of the same ecological niche (Weigel & Demuth, 2016). Nonetheless, all these changes were subtle and most probably loads below <1 % would hardly exert such an impact on gut physiology. If compared to populations modified in Trial 1 or in literature, for instance clear distress events, e.g. post-weaning period or diet change, evidenced changes were around >1 % (Frese et al., 2015; Soler et al., 2018). Also, we should take into consideration that underrepresented taxa (<1 %) depend on the sequencing depth to be detected (Ranjan et al., 2016).

6.5 Summary

The results derived from this study showed the capacity of two combinations of sodium salts of butyric or heptanoic acids, with a blend of sodium salts of medium-chain fatty acids from coconut distillates, to modulate the colonic microbiota profile, the fermentation patterns and the host response under an ETEC F4 challenge. Despite the in-feed supplementation of BUT+ was not capable of reducing the intestinal colonisation by *E. coli* F4 under our experimental conditions, the additive was able to promote some shifts in different microbial groups leading to an increase in the Firmicutes:Bacteroidetes

ratio that could be regarded as positive. Concerning HPT+ supplementation, it demonstrated ability to reduce colon colonisation by *E. coli* F4 and diminish the abundance of members of the Enterobacteriaceae family, and also it was associated to a lower colonic pH and a decrease in some fermentation products. These changes could have helped to reduce the opportunities of ETEC F4 to colonise the gut.

Chapter 7 General discussion

In the light of the results presented in this Thesis and the compilation of the papers reviewed from the literature, many factors and variables appear to condition the effects of short- and medium- chain fatty acids. With this scenario, this discussion will delve into the efficacy of the additives and the different strategies evaluated in Chapters 4, 5, and 6 to control intestinal bacterial infections. Results also will be discussed considering the limitations of animal models of disease and some thoughts will be made about the usefulness of the different parameters to be considered in this kind of models. Lastly, results obtained from the HTS of 16S rRNA gene will be discuss trying to define the characteristics of intestinal microbiota in weaning piglets and the main factors able to condition it.

7.1 Efficacy of protected sodium butyrate, protected sodium heptanoate, sodium salts of coconut MCFA or their combination on the control of intestinal bacterial infections

The additives evaluated and presented in this PhD dissertation consisted of new short- and medium-chain fatty acid (SCFA and MCFA, respectively) formulas protected with different classes of fatty acids. These new forms were conceived with the objective of improving their efficacy against enteropathogens by supporting the gut health and maturity at weaning. In Chapter 4, two new forms of sodium butyrate and sodium heptanoate protected with palm oil triglycerides (30 % of the active ingredient) were administered to piglets challenged with the most common pathogen causing the post-weaning diarrhoea, enterotoxigenic *Escherichia coli* (ETEC) F4. With this fat-protection, the bioactive compound is expected to be gradually delivered through the GIT and be capable of achieving distal segments. In most of the works reviewed that assessed protected formulas of this type of acids, the protecting agent was based on fat polymers, although most of them did not specify its chemistry. Few published works have been able to demonstrate *in vivo* the slow release of this supplemented acids by increases in their concentration in digesta (Piva et al., 2007). Most of the time authors have found inconsistent results. Willamil et al. (2011) were not capable of differentiating between (un)protected forms the presence of lactic nor formic acids in the GIT. Neither Zentek et al. (2012) that achieved to detect major concentrations of the medium-chain fatty acids

(MCFA) supplemented in the stomach of animals treated with the protected form but not in further sections of the GIT.

Also, in our case, in Chapter 4 we tried to trace both fat-protected sodium salts by measuring the levels of butyric and heptanoic acid analytes along different GIT compartments, i.e. stomach, duodenum, ileum, and colon. Butyric acid was detected in higher amounts in the stomach of the animals supplemented with butyrate, but not further on. In the case of heptanoate, it was uniquely detected in treated animals with heptanoate, in stomach and also in duodenum in reduced concentrations (<0.10 mmol/kg FM), but not further. This failure to increase the concentration of supplemented acids in the distal parts of the gut was in line of most of the former cited works. Nonetheless, some questions remain open since the analysis of these acids in punctual digesta samples of the gut could not be a method sensitive enough to evaluate the protection technology. On the other hand, as discussed in Chapter 4, if the slow release had been afforded, we would hardly detect the small aliquots being delivered. Regarding butyrate, it must be also considered as an important fermentation product in the GIT, what makes impossible to be distinguished between endogenous fermentation product and exogenous supplementation without a tracing methodology (Smith et al., 2012; van den Borne et al., 2015). Nevertheless, despite the inability of tracing protected acids along the gut, variables measured in the hindgut responded to their addition.

In general, both additives, butyrate and heptanoate, affected the entire gut fermentation as well as modified some bacterial populations in the colon, but to different extent. Butyrate showed to depresse lactic fermentation in stomach, whereas pathogen loads of *E. coli* F4 were unaffected and *E. coli* was unexpectedly increased in the ileum. Regarding these results, we hypothesised that in stomach lactobacilli may be inhibited or reduced and thus the lactic acid production, what might have allowed the favourable competence and pass of ETEC F4 and *E. coli* proliferation towards more distal parts displaying carry-over effect towards the intestines. Together to possible selective antimicrobial effect of butyrate in the stomach, it could also had exerted other effects in the gut. Actually, in the scientific community butyrate is widely acknowledged to have, rather than antimicrobial activity, tropism for the intestinal villi and support the intestinal barrier (Kien et al., 2007; Le Gall et al., 2009; Peng et al., 2009; Lacorn et al., 2010; Lewis et al., 2010; Feng et al.,

2018) and has been for long commercialised with this purpose. Therefore, in the present study, butyrate could have prevented the injury to villi of a higher number of enterobacteria and coliforms attached to intestinal mucus. Also, other interesting results observed related to the microbiota composition and functions were the depression of the intestinal fermentation and the increments of acetic acid as well as the higher presence of *Prevotella* in colon with butyrate. This could also had been due to the capacity described by other authors for some acids such as butyric acid to favour the pre-digestion of diets (Manzanilla et al., 2006; Le Gall et al., 2009) and thus reduce the available substrate arriving to the hindgut to be fermented and towards a specialised acetogenic fermentation possibly related to *Prevotella* (Holman et al., 2017). So, summarising the effects promoted by butyrate in the hindgut, these could have been derived from the changes promoted directly by butyrate in upper parts like changes in the stomach fermentation or an improved digestion of nutrients in the proximal parts of the small intestine.

Heptanoate or heptanoic acid is an uncommon compound rare in nature (De Smet et al., 2016), and in the animal nutrition field. In this context, it has been tested *in vitro* by several authors (Bhattarai et al., 2007; Grilli et al., 2013) showing good antimicrobial properties. Despite this, very few *in vivo* studies could be found evaluating heptanoate. Up to our knowledge, only one study was found in the literature (Ocasio-Vega et al., 2016) testing the same sodium heptanoate form, which increased feed efficiency due to a lower feed intake in rabbits, and also another work with pigs (De Smet et al., 2016) that found no influence of heptanoic acid in performance and almost no changes in gastric microbiota. The results in our study prompted by heptanoate consisted of trends in increasing ileal enterobacteria while *E. coli* F4 loads being unaffected in the colon, but an obvious depression of specific fermentation products in stomach in valeric, butyric and propionic acids, as well as total short-chain fatty acids (SCFA) being reduced in the colon.

Regarding effects on colonic microbiota, changes in colonic minor populations might have been involved such as the reductions registered in some populations, *Lachnospira* or *Roseburia*, or the increase of Actinobacteria, typical from the upper gut that generally produces acetate but not propionate nor butyrate (Binda et al., 2018). In this line, some inhibiting effect towards unmeasured populations in the foregut might have also been

involved and some possible carry-over effect of typical populations such as Actinobacteria from the foregut could have been enriched and reached the hindgut.

Nonetheless, another positive aspect of the use of protected forms is their ability to mask unpleasant odours, of vital importance in the adaptation of young piglets to solid diets (Partanen et al., 2002b; Partanen et al., 2007). Butyrate has been described as rancid while heptanoate as pungent (Mroz et al., 2006). Although we did not compare the protected forms against the free forms to confirm this success, feed intake was unaffected by their addition and, what is more, animals had even numerical higher average daily feed intake (ADFI) at the end of trial (day 8 PI) (Figure 7.1).

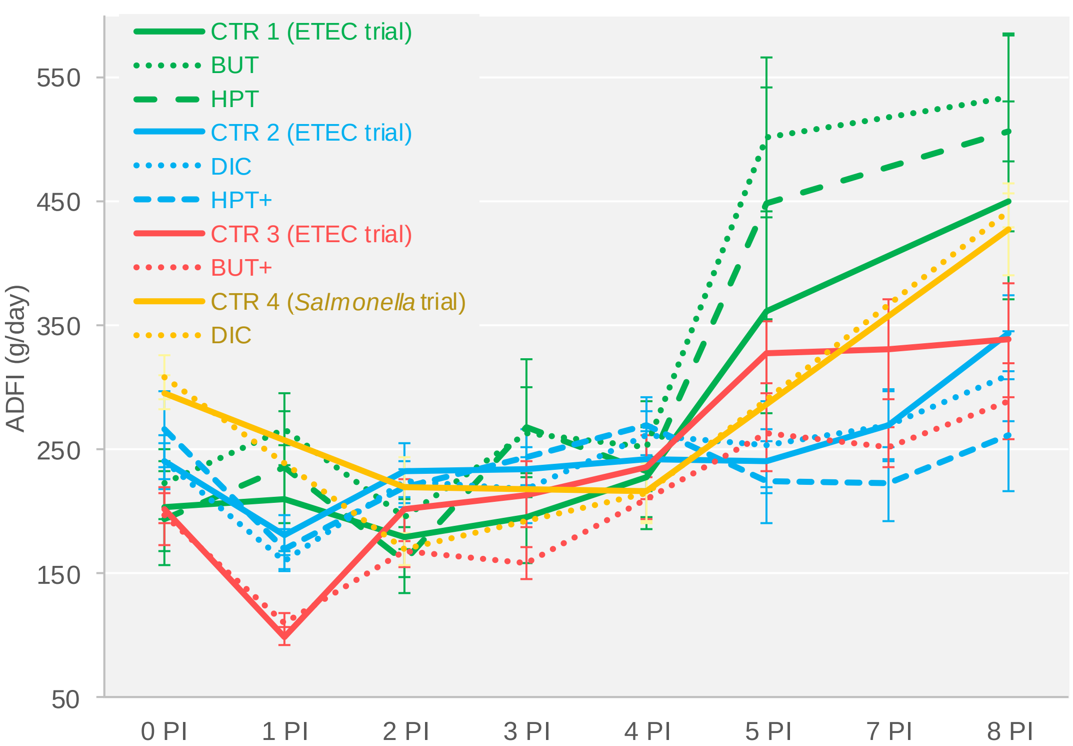


Figure 7.1 Average daily feed intake (ADFI) along the experimental period for each one of the additives tested and their respective control from Chapters 4 (CTR 1, ETEC trial), 5 (CTR 3 & 4, ETEC & *Salmonella* trials), and 6 (CTR 2 & 3, ETEC trials). Legend: CTR, control group; BUT, protected sodium butyrate; HPT, protected sodium heptanoate; DIC, sodium salts of coconut distilled MCFA mixture; BUT+, MCFA salts protecting butyrate; HPT+, MCFA salts protecting heptanoate.

Regarding the sodium salts from coconut oil distilled MCFA (minimum content of 32 % lauric acid), they were assessed in this Thesis under two pathogen challenges, *Salmonella* Typhimurium and ETEC F4, in Chapter 5. Initially, MCFA were proposed as a rapid energy

source because their rapid absorption and oxidation in the liver (Cera et al., 1990; Rodas & Maxwell, 1990; Han et al., 2011; Hong et al., 2012; Li et al., 2015; Lei et al., 2017; Weng et al., 2017) and more specifically, coconut oil has also been tested with this aim (Trevisi et al., 2017; Manzke et al., 2018). However, as with heptanoate, MCFA are more recently being extensively investigated because of their antimicrobial properties. Several studies evidenced *in vitro* their activity against common pathogens such as *Salmonella*, ETEC F4, and *Brachyspira hyodysenteriae* (Petschow et al., 1996; Bergsson et al., 2002; Batovska et al., 2009; Martínez-Vallespín et al., 2016; Vande Maele et al., 2016), especially lauric acid and monolaurin being the most effective (Skrivanová et al., 2006; Tangwatcharin & Khopaibool, 2012; Shilling et al., 2013). Moreover, some authors observed reductions of virulence factors of pathogens like *Salmonella in vitro* (Van Immerseel et al., 2004; Boyen et al., 2008b; Cox et al., 2008). Few works evaluated these properties *in vivo* against pathogens, with dissimilar outcomes. The work of Van Immerseel et al. (2004) encountered positive reductions of caecal *Salmonella* Enteritidis counts in broilers supplemented with caproic acid (C6), however, other authors met the same response because of the benefits of protecting (Boyen et al., 2008b; Molatová et al., 2011), and in the same direction others argued the lack of effects because the negligible concentrations of the acid detected in the pathogen location (Gallois et al., 2008).

Within this context, in this Thesis a consistent inhibitory effect was observed with the addition of the MCFA salts in the pathogenic populations evaluated, *Salmonella* and enterobacteria, although we were not able to detect effects on pathogenic *E. coli* F4. Unfortunately, we did not trace, for instance, lauric acid, through the GIT as in Chapter 4, and whether the effect was direct or indirect we cannot state. Regarding microbial activity along the gut, major fermentative products were unchanged, and colon microbial richness was unaffected with the MCFA salts. Regarding these variables, ecological interactions among species were interpreted. Interestingly, the reduction of some microbial groups like *Salmonella* with the MCFA salts might have allowed the progression of other potential pathogenic strains, such as Barnesiellaceae, Spirochaetaceae and Alcaligenaecae, possibly related to niche restitution. Other minor networks of cross-feeding were also hypothesised to occur under both challenges with *Fibrobacter* and Veillonellaceae members, respectively, possibly related to changes in valeric acid

amounts. Actually, these differences might have influenced the host response (Foster et al., 2017), as we observed with distinct intraepithelial lymphocytes (IEL) shifts with the MCFA supplementation between challenges. On the other hand, as with heptanoate, MCFA are described as rancid and present bad palatability (Decuypere & Dierick, 2003; Desbois & Smith, 2011), and more specifically caprylic and capric acids present goat-like odour while lauric acid presents laurel-like odour (Mroz et al., 2006). Although in this case the MCFA tested had no protection, we did not observe significant impairments in ADFI (Figure 7.1), possibly because of the salt form.

Finally, in Chapter 6 both types of additives were combined into a functional protection form of sodium butyrate and sodium heptanoate with the sodium salts of coconut distilled MCFA mixture (50 % of butyrate or heptanoate). In the literature, it was found the development, still at pilot-levels, of a MCFA-based liposome (analogous to biological membranes) (Liu et al., 2010), to be applied in feedstuffs or food. But in the animal nutrition research, up to our knowledge, none of the fat protections reported until now did test such an approach. The new formulas pretend not only to provide a protection with the MCFA salts, but to add value as a functional protection, therefore to reproduce synergistic effects. On one hand, neither butyric nor heptanoic acid analytes were differently detected in the ileum or colon of treated animals, neither MCFA were quantified. Thus, whether the protection was able to modify their concentration along de GIT cannot be confirmed. Once again, nonetheless, the addition of both additives influenced responses in the distal gut, and results from both Chapters 4 and 5 were partially summed in Chapter 6. With MCFA-protected butyrate, we hypothesised that effects seen on microbial populations and minor fermentation products might have been more driven by the action of MCFA promoting the increases of Firmicutes:Bacteroidetes (F/B) ratio in colon. Butyrate might have kept its beneficial effects for the intestinal epithelia with longer villi and increased production of mucins. These, in turn, would have left more available space for the adhesion of enterobacteria, which were increased as observed with butyrate alone. MCFA-protected butyrate also increased ileal coliforms as well as the number of animals with quantifiable *E. coli* F4.

Regarding MCFA-protected heptanoate, this additive was capable of reducing the faecal coliforms already before the challenge and consequently reduce enterobacteria and

coliforms in the gut after the infection. We also observed a lower number of animals with quantifiable levels of *E. coli* F4 in colon and simultaneously inferior percentages of colonic Enterobacteriaceae (16S rDNA sequenced) by administering the MCFA-protected heptanoate. Also, this additive reduced the fermentation in ileum and colon as observed with heptanoate. As global results, it could be hypothesised a real effective specific antimicrobial effect with the combination of different MCFA (heptanoate with mainly lauric acid), although if considering that colonic microbiota only showed minor changes, other modes of action should also be considered. These effects could have been due to improvements in the digestion in the upper parts thus limiting the available substrate reaching the hindgut or to carry-over effects of changes promoted in the microbiota of proximal parts of the gut (not analysed in this study).

Regarding effects of these combined additives in performance, and despite the limitation of the animal model to detect changes in growth rate, no one of them improved the growth of the piglets but numerically it was even worsened. Nonetheless, no negative effect was registered in the ADFI, discarding any negative effect of this kind of synergic protected forms on intake (Figure 7.1).

Summarising, the outcomes obtained in Chapter 6 with the new protected forms of butyrate and heptanoate with coconut distilled MCFA salts displayed interesting different results in front of two different ETEC F4 challenges, resembling to some extent the addition of the effects of the butyrate and heptanoate evaluated in Chapter 4 and those of MCFA salts mixture in Chapter 5. For instance, MCFA-protected butyrate was consistent with the tropic effect on intestinal epithelium and probably the enhancement of the intestinal barrier, although animals did not succeed in clearing the pathogen. In the case of MCFA-protected heptanoate, such a combination of MCFA was shown to be more effective against the pathogen and capable of preventing the raise of these populations. In this regard, the combination of heptanoate and MCFA had showed to exert the most effectivity against the pathogen, keeping the selective inhibitory effects of MCFA on enterobacteria, combined with the effects promoted by sodium heptanoate on other essential constituents of the GIT. However, more research would be needed to confirm these results, including simultaneous comparison of the individual and protected

forms under different challenging conditions, and further field studies aimed to evaluate their suitability under a natural course of post-weaning diarrhoea.

7.2 The reliability of the challenge model

For evaluating the efficacy of new antibiotic alternatives, like SCFA and MCFA in this Thesis, the use of *in vivo* appropriate challenge models represents a valuable tool. The challenge models used in Chapters 4, 5 and 6 have been far used in our group (Martín-Peláez et al., 2010; Molist et al., 2012; Gustavo Hermes et al., 2013; Guerra-Ordaz et al., 2014; Barba-Vidal et al., 2017abc) for testing diverse dietary strategies, such as dietary fibre, prebiotics, probiotics, or acid salts. ETEC F4 (post-weaning diarrhoea) and *Salmonella* Typhimurium challenge models are performed under controlled conditions and limited to one pathogen what makes them suitable for evaluating the role and mechanism of action of the additives, to assess the ability to reduce the specific infection and its consequences, like the boost of inflammatory markers or the presence of scouring. Performance has not always been responsive to ETEC challenges compared to *Salmonella* Typhimurium ones (Adewole et al., 2016). In this regard, no significant effects were registered in performance parameters with the use of the experimental diets in our trials, but the number of replicates were indeed not adequate to evaluate effects on performance. Normally, when the main objective of the study is to analyse the effect of the in-feed additives on performance, experimental designs are based in much higher number of replicates and trials are run in similar conditions to those found in the field.

It should be beard in mind that the objective of our work, therefore, rather than evaluating the effects on performance, was to study the clinical and global gut health response of the weaned piglet after the oral challenges as well as to give insights of possible modes of action. In this sense, this type of trials is integral part of the technology readiness (level) development (TRL) (NASA, 2012), whereby a product gets on the market while the technology matures; in our case the additives we tested were assessed in a relevant environment. Precisely because of this, these will have to be scaled-up, to evaluate the suitability of these strategies for instance in field conditions, where multiple pathogens can be involved, and other sanitary and management conditions influence, focused on performance as the main outcome of interest for the sector.

On the other hand, despite the tight control of variation factors between the different studies of the present Thesis (i.e. same farm of origin, same weaning age, similar plain

diet, management, pathogen strain, doses, etc.) a conflict on repeatability was observed between trials in the response of the animals to the challenge.

Although not described in the previous chapters, in each of the animal trials of this Thesis an additional group of 8 animals was included as a control for the challenge response. These additional animals received the CTR diet but were not inoculated with the pathogen but with a sterile placebo instead (placebo group, PLB). In Table 7.1 it can be followed the response of different parameters for both control group of animals (infected group -INF- and PLB). The outcomes triggered by the three challenges of ETEC F4 differed if we compared the response of the CTR treatments. In Chapter 6, when evaluating the MCFA-protected butyrate, an obvious peak of diarrhoea was observed in the CTR treatment (ETEC Trial 2), and the inflammatory biomarkers used (TNF-α or Pig-MAP) were boosted, some animals exhibiting values beyond the normal range (approx. >100 pg/mL for TNF-α and >2 mg/mL for Pig-MAP). However, when evaluating MCFA-protected heptanoate in a different ETEC challenge trial (ETEC Trial 3), animals displayed an inflammatory response, but the diarrhoea did not regress to initial values and was maintained along the trial. Animals could have suffered more stress due to manipulation, but whatever occurring from the original farm until the UAB facilities we cannot know. Also, despite animals had no symptomatology at arrival, this does not exclude the presence of other pathogens (Carpenter & Burlatschenko, 2005; Ruiz, et al., 2016).

Table 7.1 Inflammatory response and diarrhoea and rectal temperature outcomes obtained in the three different ETEC F4 trials and *Salmonella* Typhimurium trial.

		ETEC Trial 1		ETEC Trial 2		ETEC Trial 3		*Salmonella* Trial	
	Day PI	INF	PLB	INF	PLB	INF	PLB	INF	PLB
Diarrhoea[B]	0	0.79	0.13	1.70	1.63	1.13	1.27	1.33	1.44
	1	1.57	0.86	2.21	1.69	1.56	1.19	1.90	1.00
	2	1.46	1.13	1.64	1.25	1.55	1.52	3.00	1.13
	3	1.46	0.75	1.43	1.25	1.44	1.60	2.77	1.13
Rectal temperature (ºC)	0	39.4	39.1	38.9	38.6	39.0	38.6	39.4	38.9
	2	38.4	38.5	38.9	38.2	39.1	39.0	40.6	39.3
TNF-α (pg/mL)	4	111	106	129	101	156	124	312	106
Pig-MAP (mg/mL)	4	0.836	0.860	2.131	0.809	1.560	1.220	3.083	0.530

These trials are from Chapters 4 (ETEC 1 Trial), 5 (ETEC Trial 3 and *Salmonella* Trial), and 6 (ETEC Trials 2 & 3). [B] Diarrhoea score: from 0 indicating solid and firm faeces, to 4 indicating watery faeces. Legend: INF, infected group; PLB, placebo group.

In the same vein, when quantifying at the arrival of the pigs, the initial counts of faecal enterobacteria and coliforms, these were substantially different among studies with average values of enterobacteria of 9.43 ± 0.732 log CFU/g, 8.96 ± 0.630 log CFU/g, and 9.77 ± 0.282 log CFU/g fresh matter (FM) in Trial 1, 2 and 3, respectively, and of coliforms of 9.14 ± 0.818 log CFU/g, 8.02 ± 0.640 log CFU/g, and 9.70 ± 0.360 log CFU/g FM in Trial 1, 2 and 3, respectively. Also, when evaluating counts in intestinal contents, divergences were observed between trials, for instance coliforms in colon or ileal mucosal scrapes comparing Trials 2 and 3 (Figure 7.2). Also, pathogenic counts of *E. coli* F4 differed among trials (Figure 7.3). In fact, all these differences might have influenced to some extent the comparison amongst the additives.

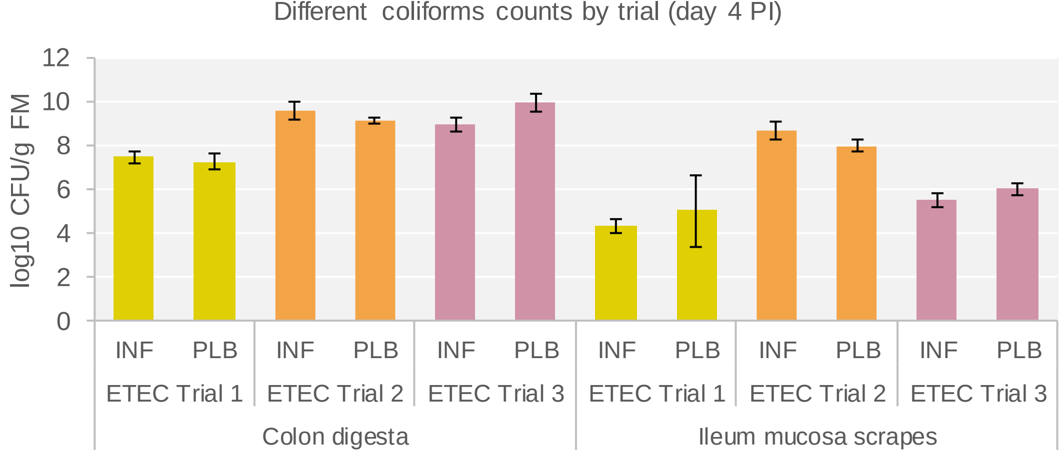


Figure 7.2 Coliform counts quantified in colon digesta and ileum mucosa scrapes in the three different ETEC trials included in Chapters 4 (ETEC 1 Trial), 5 (ETEC Trial 3), and 6 (ETEC Trials 2 & 3). Legend: INF, infected group; PLB, placebo group.

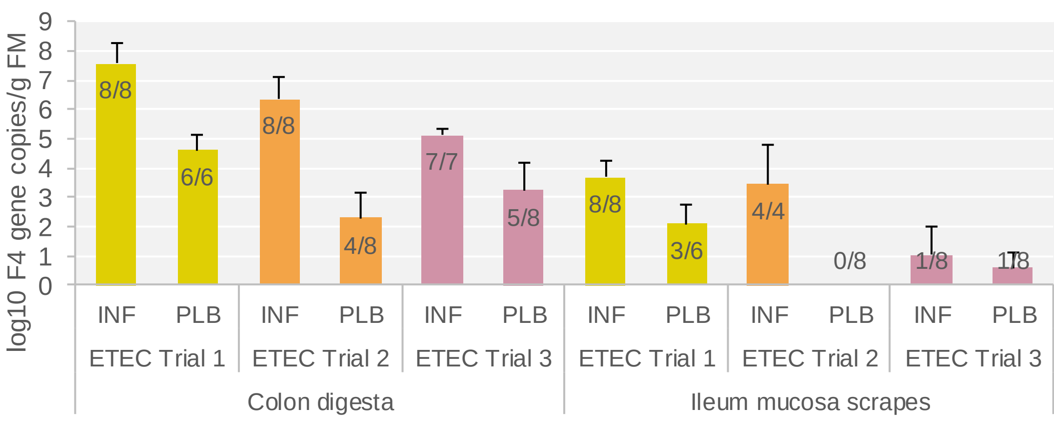


Figure 7.3 *E. coli* F4 gene copies quantified in colon digesta and ileum mucosa scrapes in the three different ETEC trials included in Chapters 4 (ETEC 1 Trial), 5 (ETEC Trial 3), and 6 (ETEC Trials 2 & 3). Legend: INF, infected group; PLB, placebo group.

7.2.1 Usefulness of the measured variables

In the studies of this Thesis a battery of parameters was included with the aim of evaluating possible modes of action of the experimental additives. Within them: microbial counts, fermentation products, histology parameters or indexes of inflammatory response like TNF-α or Pig-MAP. From all these trials, it could be possible therefore to analyse the correlation between them, and with clinical outcomes or performance, trying to identify their usefulness in this kind of challenging trials. In Table 7.2 they are presented the variables selected that may respond to the experimental treatments, and in consequence, may be inter-related. In this regard, those that usually responded consisted of: bacterial counts (enterobacteria and coliforms), histological measurements, fermentation products, and inflammatory markers. Moreover, some variables were correlated with body weight (BW). Animal BW did not show correlation with diarrhoea score. But for instance, BW was positively correlated with villous intraepithelial lymphocytes (IEL) from ileum, probably because of non-compromised animals displayed a more developed immune system (McLamb et al., 2013); actually, an activated but equilibrated immune system has to be waiting for possible external insults (Cheroutre, 2005), in line with the discussion of Chapter 5. In this sense, *Salmonella* spp. counts and *E. coli* F4 copies from colon and attached to ileum appeared to be reduced with increasing BW.

Furthermore, BW and average daily gain (ADG) were in general positively correlated with products of fermentation from colon such as ammonia, propionic, butyric, and valeric acids as well as total SCFA, but also ileum ammonia was negative correlated with *Salmonella* counts. Also, BCFA were negatively correlated with attached *E. coli* F4. Although ammonia, as BCFA, derives from protein metabolism, commonly associated to pathogens (Pieper et al., 2016), in conjunction with SCFA are products from the fermentation that increase with the evolving animal gut maturity after weaning (van Beers-Schreurs et al., 1998). In fact, SCFA are in general protectors of the milieu (den Besten et al., 2013) and this could be observed as negative correlation between colonic valeric and butyric acid with *Salmonella* counts. Another interesting factor might be the compartmentalisation of the gut, being ileum more prone to offer a hostile environment and thus forcing pathogens to develop survival factors (Looft et al., 2014a), what could

lead to hypothesise that ammonia would be aiding in this selective pressure against pathogenic strains such as *Salmonella* in this site.

Table 7.2 Correlations (Spearman correlation, ρ_S) between intestinal variables (from ileum digesta and mucosa scrapes or colon digesta) and growth and clinical performances among trials during the infectious period.

Variable 1	Variable 2	ρ_S	*P*	adj. *P*	Variable 1	Variable 2	ρ_S	*P*	adj. *P*
ileum C1	ileum C2	0.700	0	0	colon C1	colon succ	0.512	0	0
colon C4	colon C5	0.676	0	0	ADG	colon C5	0.508	0	0
ileum pH	RT	0.669	0	0	ileum lact	mucosa entero	-0.511	0	0
ADG	colon SCFA	0.654	0	0	colon C3	colon entero	-0.512	0	0
colon F4	mucosa F4	0.654	0	0	crypt	ileum C1	-0.514	0	0
ileum BCFA	mucosa F4	0.637	0	0	ADG	TNF-α	-0.521	0	0
ileum pH	colon pH	0.628	0	0	TNF-α	ileum succ	-0.534	0	0
colon C2	colon C4	0.626	0	0	ileum pH	mucosa F4	-0.552	0	0
ileum NH_3	colon NH_3	0.623	0	0	ileum lact	mucosa coli	-0.558	0	0
BW	RT	0.615	0	0	BW	mucosa F4	-0.612	0	0
BW	IEL	0.605	0	0	ileum pH	ileum BCFA	-0.632	0	0
ileum succ	ileum BCFA	0.596	0	0	BW	colon F4	-0.638	0	0
BW	colon NH_3	0.579	0	0	ADG	Pig-MAP	-0.660	0	0
ileum lact	ileum succ	0.579	0	0	ileum pH	ileum succ	-0.702	0	0
VC	ileum C1	0.577	0	0	RT	ileum succ	-0.751	0	0
ADG	colon C4	0.573	0	0	colon pH	RT	0.585	0.001	0.007
villus	colon C2	0.566	0	0	RT	TNF-α	0.545	0.001	0.007
diarrhoea	TNF-α	0.561	0	0	ileum entero	mucosa F4	0.528	0.001	0.007
BW	colon C4	0.551	0	0	TNF-α	salmonella	0.658	0.006	0.028
ileum pH	mucosa coli	0.550	0	0	VC	salmonella	-0.653	0.006	0.028
ADG	colon C3	0.547	0	0	BW	salmonella	-0.596	0.015	0.055
colon succ	colon coli	0.546	0	0	ADG	salmonella	-0.588	0.017	0.061
diarrhoea	ileum pH	0.542	0	0	colon C5	salmonella	-0.570	0.021	0.072
BW	colon SCFA	0.541	0	0	ileum NH_3	salmonella	-0.561	0.024	0.078
TNF-α	colon entero	0.541	0	0	colon C4	salmonella	-0.553	0.026	0.083
ileum pH	ileum NH_3	0.528	0	0	villus	salmonella	-0.551	0.027	0.085
ileum C1	ileum C4	0.527	0	0	Pig-MAP	salmonella	0.541	0.031	0.094
ileum lact	ileum BCFA	0.523	0	0					

Only control infected animals were considered. Only correlations ρ_S >|0.5| and adjusted *P* values <0.10 were included, as well as correlations between variables that are part of others, e.g. acetic acid within total SCFA, were excluded. Correlations are sorted by significance. Legend: ileum, ileal digesta; colon, colonic digesta; mucosa, ileal mucosa scrapings; C1, formic acid; C4, butyric acid; ADG, average daily gain; F4, *E. coli* F4 gene copies; BCFA, branched-chain fatty acid; C2, acetic acid; NH_3, ammonia; RT, rectal temperature; IEL, intraepithelial lymphocytes; BW, body weight; succ, succinic acid; lact, lactic acid; VC, villus:crypt ratio; diarrhoea, faecal score from firm (0) to watery (4) faeces; villus length, villus measured in ileum; coli, coliform counts; C3, propionic acid; SCFA, short-chain fatty acids; entero, enterobacteria counts; C5, valeric acid; crypt, crypt depth measured in ileum; salmonella, *Salmonella* spp. counts in caecum.

Moreover, with increasing *Salmonella* counts, biomarkers TNF-α and Pig-MAP also increased, while villus:crypt (VC) ratio was reduced. Actually, *Salmonella* exerts a greater acute response compared to ETEC F4 with higher inflammatory response and intestinal villus damage as shown in Chapter 5, Table 7.1, or in previous works of our group (Barba-

Vidal et al., 2017b), what probably could easily result in stronger correlated responses. Other relationships between variables were as expected, for instance clinical outcomes such are faecal score and compromised growth (lower ADG) or the raise of TNF-α. In this context, the response of such blood biomarkers makes us think that is obvious the need of useful biomarkers less-invasive that in turn can allow to follow up the animals, under more humanitarian measures. In the same vein, taking advantage of digesta samples, microbiota is also a very informative resource, despite it is complex and the interpretation of the output often is not straightforward. The intrigues of this analysis and the doubts derived from our experience will be discussed in the following section.

7.3 Gut microbiota still to be deciphered

The analysis of the colonic microbiota composition of the weaning piglets by the massive sequencing of the 16S rRNA gene provides new insights on this complex presence of bacterial and archaeal communities, furthermore, under infectious disease. Weaning associated gut microbiota is still being more extensively reviewed, with remaining changes around 2 weeks (De Rodas et al., 2018; Guevarra et al., 2018), and in parallel being described the development and maturation of the GIT (Montagne et al., 2007). On the other hand, however, those works that performed the analysis under challenge conditions such as ETEC F4 or *Salmonella* Typhimurium were in scarce numbers (Borewicz et al., 2015; Argüello et al, 2018; Bin et al., 2018, Pollock et al., 2018).

Regarding the results of our experiments, we expected to observe a broad range of response in the microbiota structure with clear dysbiotic profiles in most of the animals orally challenged that showed clinical symptoms. Actually, we took samples on day 4 PI (also on day 8 PI) expecting to observe microbiota profiles driven by the acute phase of the infection. However, the microbiota communities were quite resilient consisting mainly of Firmicutes and Bacteroidetes phyla, similarly to other weaning profiles reported in the literature (Figure 7.4). Nonetheless, not all works in the literature showed profiles with the same proportions, for instance, our profiles resembled more accurately the proportions of Firmicutes, Bacteroidetes and Proteobacteria to those obtained by Poole et al. (2013), Mach et al. (2015), and Soler et al. (2018) while those from Slifierz et al. (2013), Pajarillo et al. (2014), and Frese et al. (2015) differed to a greater extent. Age effect might have not been the cause since similar ages were compared. Other source of variation could have been the use of different methodologies, for example in the first three works QIIME was the pipeline used. Together to this, other methodological factors such as the DNA extraction kit (Smith et al., 2011; Gill et al., 2016; Lim et al., 2018), hypervariable region sequenced (Holman et al., 2017), sequencing platform (McMurdie & Holmes, 2014; Sims et al., 2014; Laudadio et al., 2018), or normalisation criteria (McMurdie & Holmes, 2014; Mallick et al., 2017; Weiss et al., 2017), as well as animal-related factors such as the breed could have been behind the differences found between authors.

Despite most of the animals evaluated in this Thesis showed a microbiota structure similar to that described in the literature for weaning piglets, in some particular animals of the first experiment within Chapter 6 (trial evaluating supplementation of MCFA-protected butyrate), we found four animals highly impaired. We assumed these animals harboured dysbiotic profiles precisely because they showed completely differentiated profiles at major taxonomic ranks, being Fusobacteria and Proteobacteria the predominant, described to predispose to post-weaning diarrhoea (Dou et al., 2017). Despite most of the authors working with challenges asserted dysbiosis, most of the results actually did not differ from "normal" weaning profiles (Figure 7.4). It could be stated that only clear differences on major taxa were observed in the study of Argüello et al. (2018), showing a clear rise of Proteobacteria in the ileal mucosa immediately two days after the pathogen inoculation rather in contents nor faeces, although animals were not excessively affected by diarrhoea, and being almost recovered 14 days after infection (Argüello et al., 2018). The same scenario was also observed in other *Salmonella* or *Lawsonia* challenges of Bowericz et al. (2015) after 2 weeks. In the light of these results, whether dysbiosis can be captured through the massive sequencing of the 16S rRNA may depend on the precision and definition of the most appropriate moment and gut location of the sampling also probably coinciding with most severe clinical signs. However, before reaching this latest stage, it still exists the possibility that animals stay resilient to external insults for instance by modulating the inflammatory-immune system in response.

In the same way, another point that should also be considered is the term *dysbiosis*, nowadays clear in concept but not fully characterised. Our expectation regarding the magnitude of changes necessary in the ecosystem to make it more vulnerable to the overgrowth of pathogens might have been overstated. It would be possible that only slight desequilibriums in certain microbial groups would be needed to be under *dysbiosis*, and that these minor groups would not be neccesarely always the same but dependant on the initial microbial ecosystem.

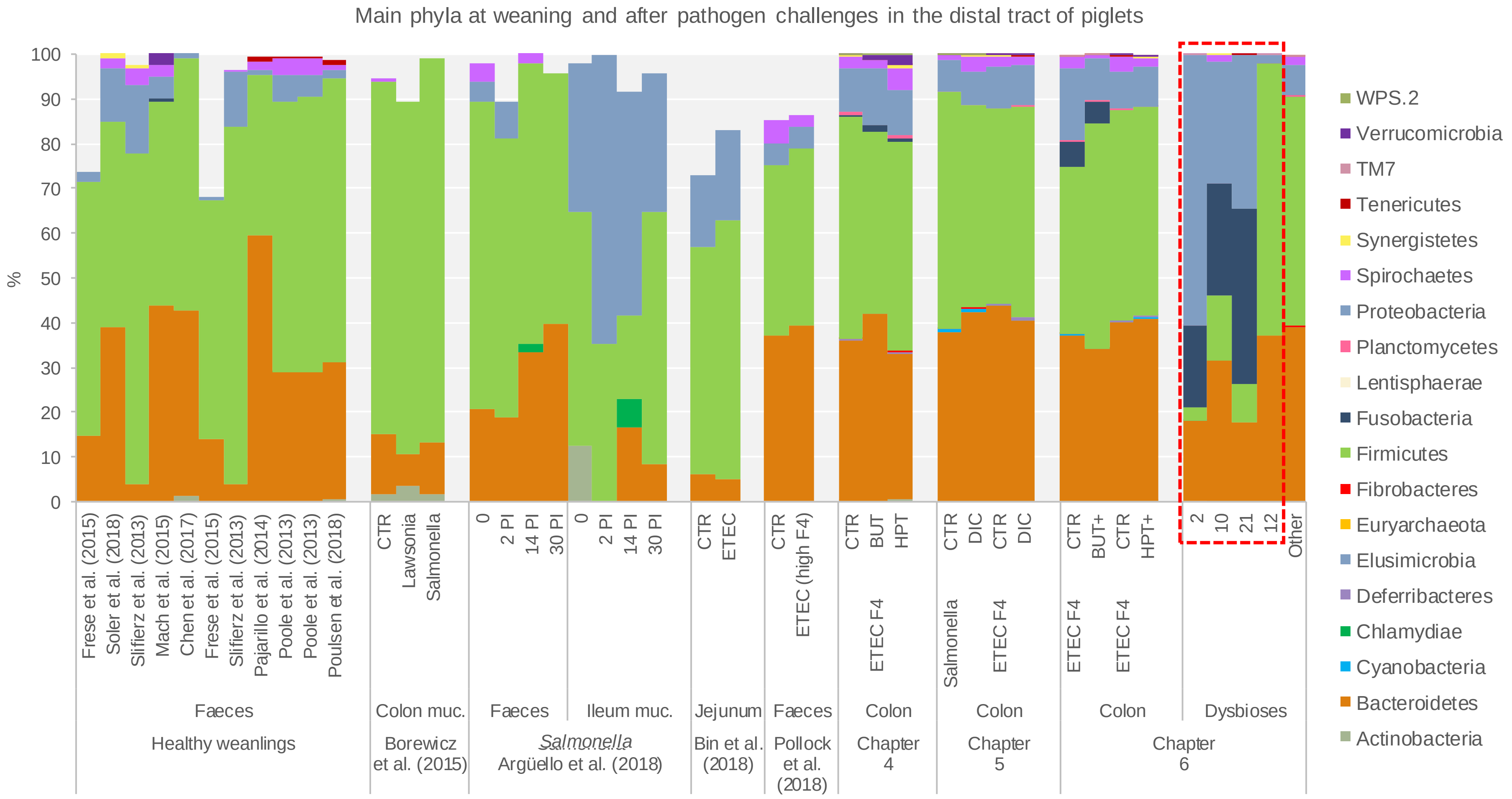


Figure 7.4 Microbiota profiles of phyla composition in healthy weaned piglets and pathogen-challenged piglets compiled from the literature, and from the results of the 16S rRNA sequencing of the colonic microbiota from Chapters 4 (ETEC Trial 1), 5 (ETEC Trial 3 & *Salmonella* Trial), and 6 (ETEC Trial 2 & 3) are presented. Also, profiles with putative dysbiosis are outlined with a dashed-line box. Legend: muc, mucosa; PI, post-inoculation days; CTR, control group; BUT, protected sodium butyrate; HPT, protected sodium heptanoate; DIC, sodium salts of coconut distilled MCFA mixture; BUT+, MCFA salts protecting butyrate; HPT+, MCFA salts protecting heptanoate.

Furthermore, we also analysed possible differences between trials in the microbiota structure trying to identify those microbial groups more sensible to be modified by uncontrolled factors. With the ordination approach (Figure 7.5), we found that studies had a significant effect on the community structure (P_{envfit} <0.001). Microbiota profiles from Chapter 4 and Chapter 6 (ETEC trial 2, testing MCFA-protected butyrate) showed the most distinctive clusters with higher differences between individuals, whereas the communities of the second study within Chapter 6 (testing MCFA-protected heptanoate) clustered together with the second experiment within Chapter 5, all under ETEC challenges, and to a lesser extent clustered with communities of animals challenged with *Salmonella* Typhimurium. Possibly *Salmonella* triggered a different community restructuration, however, animals were also weaned one week later, so age as a cofounding variable cannot be discarded. Also differences among ETEC F4 challenges could be observed, regardless of the day of sampling (days 4 or 8 PI). One possible reason that should not be discarded is that the samples from the experiment within Chapter 4 had been processed (for DNA extraction) much earlier than the rest, with slight differences in the DNA extraction protocol and also stored for a longer period in the freezer (-80 ºC) before the sequencing.

Some of the major populations (>1 %) might be indeed driving these differentiations between ETEC trials. *Fusobacterium* genus, and the phylum Fusobacteria, were enriched (P_{adj} <0.05) in the first experiment (ETEC trial 2) within Chapter 6, where the dysbiotic profiles were encountered and what might have differentiated the cluster from the rest. Other populations affected (P_{adj} <0.05) were precisely driven because of the profiles in Chapter 4, with enriched *Bacteroides*, while *Roseburia* and *Campylobacter* declined. Likewise, a sort of gradient was observed characterisng the microbiotas within Chapter 4 towards those of *Salmonella*-challenged animals, appearing the lowest proportion of *Prevotella*, and *Megasphaera* in the formers and the highest in the *Salmonella* challenge whereas the opposite occurred with *Lactobacillus* genus, enriched in animals from Chapter 4. Also, Enterobacteriaceae were enriched in the trials with clear ETEC F4 infection (Chapter 4 and ETEC trial 2 in Chapter 6).

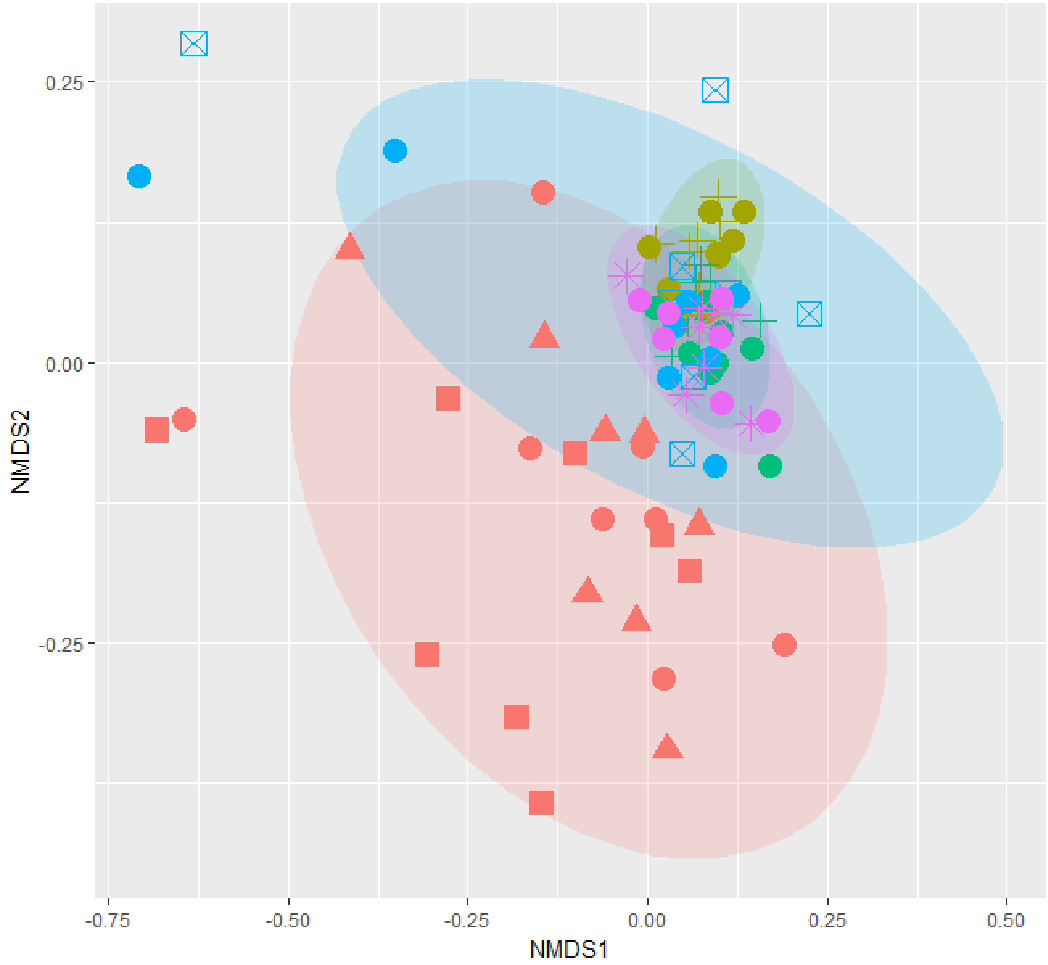


Figure 7.5 Non-metric dimensional scaling (NMDS) of the constraint disposition of the average microbial populations structure among the five studies (corresponding to a total of four trials) within Chapters 4 (● ETEC Trial 1), 5 (● *Salmonella* Trial & ● ETEC Trial 3) and 6 (● ETEC Trials 2 & ● 3). Legend: ● control diets, ▲ fat-protected sodium butyrate, ■ fat-protected sodium heptanoate, + distilled coconut MCFA salts, ⊠ MCFA-protected butyrate, and ✳ MCFA-protected heptanoate.

So, summarising, we would have had somehow microbiota repeatability among trials. Nonetheless, beyond the profile, the question remains unresolved. Which is the meaning or relevance of each one of the profiles or specific groups? Which is the functionality and the real impact on the host? Contradictory discussion can be found in literature related to this topic. For instance, Firmicutes:Bacteroidetes ratio was found to be increased in diarrhoeic piglets and was associated to the depletion of the anoxic environment (Bin et al., 2018) whereas Firmicutes is the major group present in the gut microbiota of the adult pig regarded as “beneficious” (Figure 2.13, Chapter 2) and we accepted such assumption in Chapter 6. Moreover, with this deep analysis, populations regarded as

commensals, such is *Streptococcus*, can be presented as non-harmful populations (Luise et al., 2018) or potentially pathogenic (Looft et al., 2014a), although it is obvious that a genus is constituted of hundreds of species with different imprints (Krzyściak et al., 2013). To respond to this question, few studies performed functional analysis (Looft et al., 2014a; Frese et al., 2015; Ramayo-Caldas et al., 2016), or quantified the fermentative activity to relate both variables (Argüello et al., 2018; Newman et al., 2018; Zhang et al., 2018a), giving sometimes complicated networks. Authors usually try to understand the relation among the microbial populations by a cross-feeding net of products derived from the fermentation, however, these populations can be either only presenting the gene without expressing nor producing the metabolite, just being quiescent, without need of being active. In this regard, transcript surveys of rRNA (via cDNA) could be more reliable to focus on active and viable populations (Lynch & Neufeld, 2015).

Regarding significant effects found in this Thesis in some particular minor groups (<0.01 %) (Table 7.3), they could be considered by some authors as artefacts of the methods or irrelevant results. However, these minor groups could be actually driving relevant effects in the ecosystem and play an important role in the cross-talk with the host. Ranjan et al. (2016) observed that microbiome contains a small number of abundant species, but many low abundance and rare species (<0.01 %) only representing the 3.3 % of the total abundance. Most of the effects we observed with the fat-protected butyrate and heptanoate or protected with the MCFA salts from coconut distillates were seen indeed on minor bacteria (Table 7.3). This has been a matter of discussion by other authors. Underrepresented species have been shown to make the difference between microbial ecosystems along the gut and represent the bulk of the diversity (Mann et al., 2014; De Rodas et al., 2018). Precisely, a “rare biosphere” has been described as a “seed bank” that can rise under different selective pressures and become dominant (Lynch & Neufeld, 2015). For instance, if *E. coli* F4 copies from the results seen in Chapter 4 were approximately 6 log-F4 copies/g FM in colon, and in faeces can inhabit around 10.0-11.8 log 16S rRNA gene copies of total bacteria (Stensland et al., 2015; Heinritz et al., 2016), considering the similar composition between colon and faeces (Holman et al., 2017), this infective dose in colon would represent around a 0.01 %, enough for exerting the clear diarrhoeic picture that we observed. However, this is in continuous conflict with

bioinformatical and statistical approaches when applying quality filtering or normalisation methods that may create confusion on discriminating between rare taxa or noise. Nevertheless, the abundance of some microbes can range over orders of magnitude, so deep sampling is required to detect the less-abundant members. The extensive work of Xiao et al. (2016) pointed out in this line, showing with shotgun sequencing that Firmicutes represented 28.7 % and Bacteroidetes 9.28 % of the total sequenced microbes, but the greatest percentage corresponded to members pending to be identified. In this context, we applied conservative filters presuming a more realistic scenario to evaluate the additives. If microbiota is supposed to be dynamic, we must keep a dynamic mind and accept that groups can be voluble and minor ones can suddenly take a role in the gut community. Whether the equilibrium turns towards resilience or susceptibility under specific conditions, this has yet to be deciphered.

Table 7.3 Microbial groups significantly (adjusted *P* value <0.10) modified by the experimental additives from Chapters 4, 5, and 6.

Chapter 4	Chapter 5	Chapter 6
Fat-protected butyrate	***Salmonella* challenge**	**MCFA-protected butyrate**
Synergistetes <0.5 %	Fibrobacteres <0.1 %	cand. Paraprevotellaceae ~ 6.5 %
Prevotellaceae ~ 15 %	cand. Barnesiellaceae <0.05 %	cand. *Prevotella* ~ 5 %
Helicobacteriaceae <1 %	Fibrobacteraceae <0.1 %	*Lachnobacterium* <0.5 %
Rikenellaceae <0.5 %	*Dialister* ~ 2.8 %	**MCFA-protected heptanoate**
Dethiosulfovibrionaceae <0.1 %	*Fibrobacter* <0.1 %	WCHB1-25 <0.05 %
cand. Barnesiellaceae <0.5 %	*Lachnobacterium* <0.01 %	Peptococcaceae <0.05 %
Streptococcaceae <0.01 %	*Butyrivibrio* <0.5 %	Enterobacteriaceae <0.5 %
Anaerovibrio ~ 2.5 %	**ETEC F4 challenge**	Pasteurellaceae <1 %
Bilophila <0.5 %	*Dialister* <0.05 %	Turicibacteriaceae <0.5 %
Prevotella ~ 15 %	*Lachnospira* <1 %	*Lachnobacterium* <0.5 %
Paraprevotella <0.1 %		*Aggregatibacter* <0.05 %
Dialister <0.05 %		
Helicobacter <1 %		
Streptoccus <0.01 %		
Fat-protected heptanoate		
Actinobacteria <0.5 %		
Coriobacteriaceae <0.5 %		
Paraprevotella <0.1 %		
Lachnospira <1 %		
Roseburia ~ 1.3 %		
Oxalobacter <0.01 %		
Parabacteroides ~ 1.1 %		
Collinsella <0.1 %		

Minor groups were considered <1 %, and abundance was reported if lower than 0.01, 0.05, 0.01, 0.1, 0.5 or 1 %.

Chapter 8 Conclusions

1. The dietary supplementation of fat-protected sodium butyrate at 0.3 % to weaned piglets, orally challenged with enterotoxigenic *Escherichia coli* (ETEC) F4 depresses lactic fermentation in stomach and reduces short-chain fatty acids (SCFA) concentration in colon. Microbial activity is changed towards a more acetogenic fermentation. These changes are concomitant with increases of enterobacteria and coliforms in ileum and the rise of *Prevotella* in colon and the trend for a lower Firmicutes:Bacteroidetes (F/B) ratio (P = 0.11). No change is promoted in the intestinal colonisation by *E. coli* F4.
2. The dietary supplementation of fat-protected sodium heptanoate at 0.3 % to ETEC F4 challenged piglets modifies the fermentative patterns of the stomach and promotes a decrease of SCFA concentration in colon. Heptanoate also tends to increase enterobacteria and coliforms in ileum with a slight impact on colonic microbiota with changes in Actinobacteria and some Clostridiales and Bacteroidales. No change is promoted in the intestinal colonisation by *E. coli* F4.
3. The addition of sodium salts of coconut distilled medium-chain fatty acids (MCFA) at 0.3 % to weanling piglets orally challenged with *Salmonella* Typhimurium or ETEC F4 is able to reduce counts of *Salmonella* spp. and enterobacteria in the hindgut, although *E. coli* F4 remains unaffected. The additive also promotes changes in colonic microbiota depending on the pathogen challenge. Under *Salmonella* challenge, the ratio F/B ratio tends to decrease (P = 0.13) and the minor phylum Fibrobateres is enriched, whereas under the ETEC challenge, these MCFA salts shift the global microbial communitie's structure whereby *Dialister* is promoted as well as the corresponding major family, Veillonellaceae, shows trends in the same direction (P = 0.14).
4. Supplementing sodium butyrate protected with coconut distilled MCFA at 0.3 % in the diets of weaned piglets orally challenged with ETEC F4 is not able to reduce *E. coli* F4 colonisation, but even increasing ileal enterobacteria, associated to the boost of serum TNF-α. The additive favours the increase of F/B ratio in colon (P = 0.05) and of the number of goblet cells in ileum.
5. When supplementing sodium heptanoate protected with coconut distilled MCFA at 0.3 % to ETEC F4-challenged piglets, it exerts consistent antimicrobial effects against the pathogen, being capable of reducing the presence of *E. coli* F4 as well

as counts of Enterobacteriaceae, measured by the sequencing of the 16S rRNA gene. With this additive, decreases on total SCFA are also observed in colon and an impact on ileal architecture by reducing the number of goblet cells.

6. The colonic microbiota analysis by high-throughput sequencing of the 16S rRNA gene shows that the oral challenges to weaning pigs with *Salmonella* Typhimurium or ETEC F4 do not promote big changes in the microbiota structure. Firmicutes and Bacteroidetes are shown to be the major phyla as commonly described at weaning.
7. In the same vein, the changes promoted by the tested short- and medium-chain fatty acids are most of the time restricted to minor populations, demonstrating the high resilience of colonic microbiota of weaning piglets.

Chapter 9 References

Acosta, E. (2008). Testing the effectiveness of nutrient delivery systems. In N. Garti (Eds,), *Delivery and controlled release of bioactives in foods and nutraceuticals* (1st ed., pp. 53-106). Cambridge, UK: Woodhead Publishing Ltd.

Adewole, D. I., Kim, I. H., & Nyachoti, C. M. (2016). Gut health of pigs: Challenge models and response criteria with a critical analysis of the effectiveness of selected feed additives - A review. *Asian-Australasian Journal of Animal Science*, 29(7), 909-924. doi:10.5713/ajas.15.0795

AEMPS. (2015, September). Plan Nacional de Resistencia a Antibióticos. Plan estratégico y de acción para reducir el riesgo de selección y diseminación de la resistencia a los antibióticos. Retrieved from http://www.resistenciaantibioticos.es/es/system/files/content_images/plan_nacional_resistencia_antibioticos.pdf

Allaire, J. J., Gandrud, C., Russell, K., & Yetman, C. J. (2017). networkD3: D3 JavaScript network graphs from R. Retrieved from https://CRAN.R-project.org/package=networkD3

Almeida, L. R., Costa, P. S., Nascimento, A. M. A., Reis, M. P., Barros, K. O., Alvim, L. B., . . . de Moura, S. B. (2018). Porcine stomachs with and without gastric ulcer differ in *Lactobacillus* load and strain characteristics. *Canadian Journal of Microbiology*, 64(7), 493-499. doi:10.1139/cjm-2017-0758

AOAC International. (2007). Official methods of analysis (18th ed. 2nd rev. ed.). Gaithersburg, MD: Association of Official Analytical Chemists.

Apajalahti, J., Rademacher, M., Htoo, J. K., Redshaw, M., & Kettunen, A. (2009). Divergent modulation of swine ileal microbiota by formic acid and methionine hydroxy analogue-free acid. *Animal*, 3(6), 817-825. doi:10.1017/S1751731109004431

Argenzio, R. A., & Southworth, M. (1975). Sites of organic acid production and absorption in gastrointestinal tract of the pig. *The American Journal of Physiology*, 228(2), 454-460. doi:10.1152/ajplegacy.1975.228.2.454

Argüello, H., Carvajal, A., Costillas, S., & Rubio, P. (2013). Effect of the addition of organic acids in drinking water or feed during part of the finishing period on the prevalence of *Salmonella* in finishing pigs. *Foodborne Pathogens and Disease*, 10(10), 842-849. doi:10.1089/fpd.2013.1497

Argüello, H., Estellé, J., Zaldívar-López, S., Jiménez-Marín, Á., Carvajal, A., López-Bascón, M. A., . . . Garrido, J. J. (2018). Early *Salmonella* Typhimurium infection in pigs disrupts microbiome composition and functionality principally at the ileum mucosa. *Scientific Reports*, 8, 7788. doi:10.1038/s41598-018-26083-3

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., . . . Bork, P. (2011). Enterotypes of the human gut microbiome. *Nature*, 473(7346), 174-180. doi:10.1038/nature09944

AVEKA. (2019). Spray drying. Retrieved from https://www.aveka.com/processing/spray-drying/

Bae, Y. M., & Lee, S. Y. (2017). Effect of salt addition on acid resistance response of *Escherichia coli* O157:H7 against acetic acid. *Food Microbiology*, 65, 74-82. doi:10.1016/j.fm.2016.12.021

Bailey, M., Haverson, K., Inman, C., Harris, C., Jones, P., Corfield, G., Miller, B., & Stokes, C. (2005). The development of the mucosal immune system pre- and post-weaning: Balancing regulatory and effector function. *The Proceedings of the Nutririon Society*, 64(4), 451-457.

Baltić, B., Starčević, M., Đorđević, J., Mrdović, B., & Marković, R. (2017). Importance of medium chain fatty acids in animal nutrition. *IOP Conference Series: Earth and Environmental Science*, 85. doi:10.1088/1755-1315/85/1/012048

Barba-Vidal, E., Buttow Roll, V. F., Castillejos, L., Guerra-Ordaz, A. A., Manteca, X., Mallo, J. J., & Martín-Orúe, S. M. (2017a). Response to a *Salmonella* Typhimurium challenge in piglets supplemented with protected sodium butyrate or *Bacillus licheniformis*: effects on performance, intestinal health and behavior. *Translational Animal Science*, 1(2), 186-200. doi:10.2527/tas2017.0021

Barba-Vidal, E., Castillejos, L., López-Colom, P., Rivero Urgell, M., Moreno Muñoz, J. A., & Martín-Orúe, S. M. (2017b). Evaluation of the probiotic strain *Bifidobacterium longum* subsp. *infantis* CECT 7210 capacities to improve health status and fight digestive pathogens in a piglet model. *Frontiers in Microbiology*, 11(8), 533. doi:10.3389/fmicb.2017.00533

Barba-Vidal, E., Buttow Roll, V. F., Manzanilla, E. G., Torrente, C., Moreno Muñoz, J. A., Pérez, J. F., & Martín-Orúe, S. M. (2017c). Blood parameters as biomarkers in a *Salmonella* spp. disease model of weaning piglets. *PLoS One*, 12(10), e0186781. doi:10.1371/journal.pone.0186781

Barrow, P. A., Fuller, R., & Newport, M. J. (1977). Changes in the microflora and physiology of the anterior intestinal tract of pigs weaned at 2 days, with special reference to the pathogenesis of diarrhea. *Infection and Immunity*, 18(3), 586-595.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models using lme4. *Journal of Statistical Software*, 67(1), 1-48. doi:10.18637/jss.v067.i01

Batonon-Alavo, D. I., Bouza, B., Cholet, J. C. G., & Mercier, Y. (2016). A method for determination of the acidifying value of organic acids used in pig diets in the acid binding capacity at pH 4 (ABC-4) system. *Animal Feed Science and Technology*, 216, 197-203. doi:10.1016/j.anifeedsci.2016.03.021

Batovska, D. I., Todorova, I. T., Tsvetkova, I. V., & Najdenski, H. M. (2009). Antibacterial study of the medium chain fatty acids and their 1-monoglycerides: Individual effects and synergistic relationships. *Polish Journal of Microbiology*, 58(1), 43-47.

Bearson, S. M., Allen, H. K., Bearson, B. L., Looft, T., Brunelle, B. W., Kich, J. D., . . . Stanton, T. B. (2013). Profiling the gastrointestinal microbiota in response to *Salmonella*: low versus high *Salmonella* shedding in the natural porcine host. *Infection, Genetics and Evolution*, 16, 330-340. doi:10.1016/j.meegid.2013.03.022

Bedford, A., & Gong, J. (2018). Implications of butyrate and its derivatives for gut Health and animal production. *Animal Nutrition*, 4(2), 151-159. doi:10.1016/j.aninu.2017.08.010

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*, 57(1), 289-300.

Benshitrit, R. C., Levi, C. S., Tal, S. L., Shimoni, E., & Lesmes, U. (2012). Development of oral food-grade delivery systems: Current knowledge and future challenges. *Food & Function*, 3(1), 10-21. doi:10.1039/c1fo10068h

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews*, 70(2), 567-590. doi:10.1152/physrev.1990.70.2.567

Bergsson, G., Steingrímsson, Ó., & Thormar, H. (2002). Bactericidal effects of fatty acids and monoglycerides on Helicobacter pylori. *International Journal of Antimicrobial Agents*, 20(4), 258-262. doi:10.1016/S0924-8579(02)00205-4

Bhandari, S. K., Xu, B., Nyachoti, C. M., Giesting, D. W., & Krause, D. O. (2008). Evaluation of alternatives to antibiotics using an *Escherichia coli* K88+ model of piglet diarrhea: Effects on gut microbial ecology. *Journal of Animal Science*, 86(4), 836-847. doi:10.2527/jas.2006-822

Bhattarai, H. D., Paudel, B., Park, N. S., Lee, K. S., & Shin, H. W. (2007). Evaluation of antifouling activity of eight commercially available organic chemicals against the early foulers marine bacteria and *Ulva* spores. *Journal of Environmental Biology*, 28(4), 857-863.

Biagi, G., Piva, A., Moschini, M., Vezzali, E., & Roth, F. X. (2007). Performance, intestinal microflora, and wall morphology of weanling pigs fed sodium butyrate. *Journal of Animal Science*, 85(5), 1184-1191. doi:10.2527/jas.2006-378

Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E. G., Mackie, R., . . . Zhu, W. (2016). Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environmental Microbiology*, 18(5), 1566-1577. doi:10.1111/1462-2920.13272

Bin, P., Tang, Z., Liu, S., Chen, S., Xia, Y., Liu, J., . . . Zhu, G. (2018). Intestinal microbiota mediates enterotoxigenic *Escherichia coli*-induced diarrhea in piglets. *BMC Veterinary Research*, 14(1), 385. doi:10.1186/s12917-018-1704-9

Binda, C., Lopetuso, L. R., Rizzatti, G., Gibiino, G., Cennamo, V., & Gasbarrini, A. (2018). Actinobacteria: A relevant minority for the maintenance of gut homeostasis. *Digestive and Liver Disease*, 50(5), 421-428. doi:10.1016/j.dld.2018.02.012

Blaut, M., Collins, M. D., Welling, G. W., Doré, J., van Loo, J., & de Vos, W. (2002). Molecular biological methods for studying the gut microbiota: the EU human gut flora project. *British Journal of Nutrition*, 87(S2), S203-S211. doi:10.1079/BJNBJN/2002539

Boas, A. D. C. V., Budiño, F. E. L., Neto, M. A. T., Schmidt, A., Dadalt, J. C., Monferdini, R. P., . . . Pizzolante, C. C. (2016). Organic acids in diets of weaned piglets: Performance, digestibility and economical viability. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 68(4), 1015-1022. doi:10.1590/1678-4162-8501

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., . . . Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods*, 10(1), 57-59. doi:10.1038/nmeth.2276

Borewicz, K. A., Kim, H. B., Singer, R. S., Gebhart, C. J., Sreevatsan, S., Johnson, T., & Isaacson, R. E. (2015). Changes in the porcine intestinal microbiome in response to infection with *Salmonella*

enterica and *Lawsonia intracellularis*. *PLoS One*, 10(10), e0139106. doi:10.1371/journal.pone.0139106

Bosi, P., Sarli, G., Casini, L., De Filippi, S., Trevisi, P., Mazzoni, M., & Merialdi, M. (2005). Effect of dietary addition of free or fat-protected calcium formate n growth, intestinal morphology and health of *Escherichia coli* k88 challenged weaning pigs. *Italian Journal of Animal Science*, 4(2), 452-454. doi:10.4081/ijas.2005.2s.452

Bosi, P., Mazzoni, M., De Filippi, S., Trevisi, P., Casini, L., Petrosino, G., & Lalatta-Costerbosa, G. (2006). A continuous dietary supply of free calcium formate negatively affects the parietal cell population and gastric RNA expression for H^+/K^+-ATPase in weaning pigs. *The Journal of Nutrition*, 136(5), 1229-1235. doi:10.1093/jn/136.5.1229

Bosi, P., Sarli, G., Casini, L., De Filippi, S., Trevisi, P., Mazzoni, M., & Merialdi, G. (2007). The influence of fat protection of calcium formate on growth and intestinal defence in *Escherichia coli* K88-challenged weanling pigs. *Animal Feed Science and Technology*, 139(3-4), 170-185. doi:10.1016/j.anifeedsci.2006.12.006

Bosi, P., Messori, S., Nisi, I., Russo, D., Casini, L., Fabio, F., . . . Trevisi, P. (2009). Effect of different butyrate supplementations on growth and health of weaning pigs challenged or not with *E. coli* K88. *Italian Journal of Animal Science*, 8(S2), 268-270. doi:10.4081/ijas.2009.s2.268

Boyen, F., Haesebrouck, F., Maes, D., Van Immerseel, F., Ducatelle, R., & Pasmans, F. (2008a). Non-typhoidal *Salmonella* infections in pigs: A closer look at epidemiology, pathogenesis and control. *Veterinary Microbiology*, 130(1-2), 1-19. doi:10.1016/j.vetmic.2007.12.017

Boyen, F., Haesebrouck, F., Vanparys, A., Volf, J., Mahu, M., Van Immerseel, F., . . . Pasmans, F. (2008b). Coated fatty acids alter virulence properties of *Salmonella* Typhimurium and decrease intestinal colonization of pigs. *Veterinary Microbiology*, 132(3-4), 319-327. doi:10.1016/j.vetmic.2008.05.008

Boyen, F., Pasmans, F., Van Immerseel, F., Donné, E., Morgan, E., Ducatelle, R., & Haesebrouck, F. (2009). Porcine *in vitro* and *in vivo* models to assess the virulence of *Salmonella enterica* serovar Typhimurium for pigs. Laboratory Animals, 43(1), 46-52. doi:10.1258/la.2007.007084

Bugaut, M. (1987). Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. Comparative *Biochemistry and Physiology*, 86(3), 439-472. doi:10.1016/0305-0491(87)90433-0

Burbach, K., Seifert, J., Pieper, D. H., & Camarinha-Silva, A. (2016). Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions. *MicrobiologyOpen*, 5(1), 70-82. doi:10.1002/mbo3.312

Burdet, C., Sayah-Jeanne, S., Nguyen, T. T., Hugon, P., Sablier-Gallis, F., Saint-Lu, N., . . . de Gunzburg, J. (2018). Antibiotic-induced dysbiosis predicts mortality in an animal model of *Clostridium difficile* infection. *Antimicrobial Agents and Chemotherapy*, 62(10), e00925-18. doi:10.1128/AAC.00925-18

Calveyra, J. C., Nogueira, M. G., Kich, J. D., Biesus, L. L., Vizzotto, R., Berno, L., . . . Cardoso, M. (2012). Effect of organic acids and mannanoligosaccharide on excretion of *Salmonella*

typhimurium in experimentally infected growing pigs. *Research in Veterinary Science*, 93(1), 46-47. doi:10.1016/j.rvsc.2011.08.018

Canibe, N., Steien, S. H., Øverland, M., & Jensen, B. B. (2001). Effect of K-diformate in starter diets on acidity, microbiota, and the amount of organic acids in the digestive tract of piglets, and on gastric alterations. *Journal of Animal Science*, 79(8), 2123-2133.

Canibe, N., & Jensen, B. B. (2003). Fermented and nonfermented liquid feed to growing pigs: effect on aspects of gastrointestinal ecology and growth performance. *Journal of Animal Science*, 81(8), 2019-2031. doi:10.2527/2003.8182019x

Canibe, N., Højberg, O., Højsgaard, S., & Jensen, B. B. (2005). Feed physical form and formic acid addition to the feed affect the gastrointestinal ecology and growth performance of growing pigs. *Journal of Animal Science*, 83(6), 1287-1302. doi:10.2527/2005.8361287x

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., . . . Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7(5), 335-336. doi:10.1038/nmeth.f.303

Carlson, S. A., Barnhill, A. E., & Griffith, R. W. (2012). Salmonellosis. In J. J. Zimmerman, L. A. Karriker, A. Ramirez, K. J. Schwartz, & G. W. Stevenson (Eds.), *Diseases of swine* (10th ed., pp. 821-833). Chichester, UK: Wiley-Blackwell.

Carpenter, J. A., & Burlatschenko, S. (2005). Diarrhea in nursery pigs associated with multiple concurrent pathogens. *Journal of Swine Health and Production*, 13(4), 218-221.

Casanova-Higes, A., Andrés-Barranco, S., & Mainar-Jaime, R. C. (2017). Effect of the addition of protected sodium butyrate to the feed on *Salmonella* spp. infection dynamics in fattening pigs. *Animal Feed Science and Technology*, 231, 12-18. doi:10.1016/j.anifeedsci.2017.06.008

Castillo, M., Martín-Orúe, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Pérez, J. F., & Gasa, J. (2006). The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. *Journal of Animal Science*, 84(10), 2725-2734. doi:10.2527/jas.2004-556

Cera, K. R., Mahan, D. C., & Reinhart, G. A. (1990). Evaluation of various extracted vegetable oils, roasted soybeans, medium-chain triglyceride and an animal-vegetable fat blend for postweaning swine. *Journal of Animal Science*, 68(9), 2756-2765.

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics*, 11(4), 265-270.

Chen, L., Xu, Y., Chen, X., Fang, C., Zhao, L., & Chen, F. (2017). The maturing development of gut microbiota in commercial piglets during the weaning transition. *Frontiers in Microbiology*, 8, 1688. doi:10.3389/fmicb.2017.01688

Cheroutre, H. (2005). IELs: enforcing law and order in the court of the intestinal epithelium. *Immunological Reviews*, 206, 114-131. doi:10.1111/j.0105-2896.2005.00284.x

Cheroutre, H., & Madakamutil, L. (2005). Mucosal effector memory T cells: The other side of the coin. *Cellular and Molecular Life Sciences*, 62(23), 2853-2866. doi:10.1007/s00018-005-5232-y

Cherrington, C. A., Hinton, M., Mead, G. C., & Chopra, I. (1991). Organic Acids: Chemistry, antibacterial activity and practical applications. *Advances in Microbial Physiology*, 32, 87-108. doi:10.1016/S0065-2911(08)60006-5

Christie, P. J., & Dunny, G. M. (1984). Antibiotic selection pressure resulting in multiple antibiotic resistance and localization of resistance determinants to conjugative plasmids in streptococci. *Journal of Infectious Diseases*, 149(1), 74-82. doi:10.1093/infdis/149.1.74

Claus, R., Günthner, D., & Letzguss, H. (2007). Effects of feeding fat-coated butyrate on mucosal morphologyand function in the small intestine of the pig. *Journal of Animal Physiology and Animal Nutrition*, 91(7-8), 312-318. doi:10.1111/j.1439-0396.2006.00655.x

Clouard, C., & Val-Laillet, D. (2014). Impact of sensory feed additives on feed intake, feed preferences, and growth of female piglets during the early postweaning period. *Journal of Animal Science*, 92(5), 2133-2140. doi:10.2527/jas2013-6809

Cohan, F. M., & Koeppel, A. F. (2008). The origins of ecological diversity in prokaryotes. *Current Biology*, 18(21), 1024-1034. doi:10.1016/j.cub.2008.09.014

Cole, D. J., Beal, R. M., & Luscombe, J. R. (1968). The effect on performance and bacterial flora of lactic acid, propionic acid, calcium propionate and calcium acrylate in the drinking water of weaned pigs. *Veterinary Research*, 83(18), 459-464. doi:10.1136/vr.83.18.459

Cooke, A. R. (1975). Control of gastric emptying and motility. *Gastroenterology*, 68(4), 804-816. doi:10.1016/S0016-5085(75)80295-2

Coussement, P. A. (2002). Communicating about gut health to the consumer: presenting the BENEO Programme. *British Journal of Nutrition*, 87(S2), S301-S303. doi:10.1079/BJN/2002553

Cox, A. B., Rawlison, L. A., Baird, A. W., Bzik, V., & Brayden, D. J. (2008). *In vitro* interactions between the oral absorption promoter, sodium caprate (C(10)) and *S. typhimurium* in rat intestinal ileal mucosae. *Pharmaceutical Research*, 25(1), 114-122. doi:10.1007/s11095-007-9354-9

Cramwell, P. D. (1985). The development of acid and pepsin (EC 3.4.23.1) secretory capacity in the pig; the effects of age and weaning. 1. Studies in anaesthetized pigs. *British Journal of Nutrition*, 54(1), 305-320.

Creus, E., Pérez, J. F., Peralta, B., Baucells, F., & Mateu, E. (2007). Effect of acidified feed on the prevalence of *Salmonella* in market-age pigs. *Zoonoses and Public Health*, 54(8), 314-319. doi:10.1111/j.1863-2378.2007.01069.x

Cuche, G., & Malbert, C. H. (1999). Short-chain fatty acids present in the ileum inhibit fasting gastrointestinal motility in conscious pigs. *Neurogastroenterology and Motility*, 11(3), 219-225. doi:10.1046/j.1365-2982.1999.00149.x

Cuche, G., Cuber, J. C., & Malbert, C. H. (2000). Ileal short-chain fatty acids inhibit gastric motility by a humoral pathway. *American Journal of Physiology*. *Gastrointestal and Liver Physiology*, 279(5), G925-G930. doi:10.1152/ajpgi.2000.279.5.G925

Cuche, G., Blat, S., & Malbert, C. H. (2001). Desensitization of ileal vagal receptors by short-chain fatty acids in pigs. *American Journal of Physiology. Gastrointestal and Liver Physiology*, 280(5), G1013-G1021.

D'Aoust, J. Y., & Maurer, J. (2007). *Salmonella* species. In M. P. Doyle, L. R. Beuchat, & T. J. Montville (Eds.), *Food microbiology: Fundamentals and frontiers* (3rd ed., pp. 187-236). Washington, DC: ASM Press.

De Ridder, L., Maes, D., Dewulf, J., Pasmans, F., Boyen, F., Haesebrouck, F., . . . Van der Stede, Y. (2013a). Evaluation of three intervention strategies to reduce the transmission of *Salmonella* Typhimurium in pigs. *The Veterinary Journal*, 197(3), 613-618. doi:10.1016/j.tvjl.2013.03.026

De Ridder, L., Maes, D., Dewulf, J., Pasmans, F., Boyen, F., Haesebrouck, F., . . . Van der Stede, Y. (2013b). Effect of a DIVA vaccine with and without in-feed use of coated calcium-butyrate on transmission of *Salmonella* Typhimurium in pigs. *BMC Veterinary Research*, 9, 243. doi:10.1186/1746-6148-9-243

De Rodas, B., Youmans, B. P., Danzeisen, J. L., Tran, H., & Johnson, T. J. (2018). Microbiome profiling of commercial pigs from farrow to finish. *Journal of Animal Science*, 96(5), 1778-1794. doi:10.1093/jas/sky109

De Smet, S., Michiels, J., Ovyn, A., Dierick, N., Laget, M., Cools, A., & Lauwaerts, A. (2016). Gut antibacterial effects of C7 and C9 carboxylic acids in the diet of piglets. *Journal of Animal Science*, 94(S3), 54-57. doi:10.2527/jas2015-9532

DebMandal, M., & Mandal, S. (2011). Coconut (*Cocos nucifera* L.: Arecaceae): in health promotion and disease prevention. *Asian Pacific Journal of Tropical Medicine*, 4(3), 241-247. doi:10.1016/S1995-7645(11)60078-3

Decuypere, J. A., & Dierick, N. A. (2003). The combined use of triacylglycerols containing medium-chain fatty acids and exogenous lipolytic enzymes as an alternative to in-feed antibiotics in piglets: concept, possibilities and limitations. An overview. *Nutrition Research Reviews*, 16(2), 193-210. doi:10.1079/NRR200369

Deminicis, R. G. S., Mendonça, P. P., Paula, M. O., Deminicis, B. B., & Moreira, Y. R. (2017). Supplementation with coconut oil for piglets until weaning. *Archivos de Zootecnia*, 66(255), 443-448.

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, 54(9), 2325-2340. doi:10.1194/jlr.R036012

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., . . . Andersen, G. L. (2006). Greengenes, a chimera-checked 16s rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, 72(7), 5069-5072. doi:10.1128/AEM.03006-05

Desbois, A. P., & Smith, V. J. (2010). Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology*, 85(6), 1629-1642. doi:10.1007/s00253-009-2355-3

Devi, S. M., & Kim, I. H. (2014). Effect of medium chain fatty acids (MCFA) and probiotic (*Enterococcus faecium*) supplementation on the growth performance, digestibility and blood profiles in weanling pigs. *Veterinarni Medicina*, 59(11), 527-535

Devi, S. M., Lee, K. Y., & Kim, I. H. (2016). Analysis of the effect of dietary protected organic acid blend on lactating sows and their piglets. *Revista Brasileira de Zootecnia*, 45(2). doi:10.1590/S1806-92902016000200001

Dibner, J. J., & Buttin, P. (2002). Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. *Journal of Applied Poultry Research*, 11(4), 453-463. doi:10.1093/japr/11.4.453

Dierick, N. A., Decuypere, J. A., Molly, K., Van Beek, E., & Vanderbeke, E. (2002a). The combined use of triacylglycerols (TAGs) containing medium chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition. II. *In vivo* release of MCFAs in gastric cannulated and slaughtered piglets by endogenous and exogenous lipases; effects on the luminal gut flora and growth performance. *Livestock Production Science*, 76(1-2), 1-16. doi:10.1016/S0301-6226(01)00331-1

Dierick, N. A., Decuypere, J. A., Molly, K., Van Beek, E., & Vanderbeke, E. (2002b). The combined use of triacylglycerols containing medium-chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition. I. *In vitro* screening of the release of MCFAs from selected fat sources by selected exogenous lipolytic enzymes under simulated pig gastric conditions and their effects on the gut flora of piglets. *Livestock Production Science*, 75(2), 129-142. doi:10.1016/S0301-6226(01)00303-7

Dierick, N. A., Decuypere, J. A., & Degeyter, I. (2003). The combined use of whole *Cuphea* seeds containing medium chain fatty acids and an exogenous lipase in piglet nutrition. *Archiv für Tierernährung*, 57(1), 49-63. doi:10.1080/0003942031000086626

Dierick, N. A., Michiels, J., & Van Nevel, C. (2004). Effect of medium chain fatty acids and benzoic acid, as alternatives for antibiotics, on growth and some gut parameters in piglets. *Communications in Agricultural and Applied Biological Sciences*, 69(2), 187-190.

Dong, D., Ni, Q., Wang, C., Zhang, L., Li, Z., Jiang, C., . . . Peng, Y. (2018). Effects of intestinal colonization by *Clostridium difficile* and *Staphylococcus aureus* on microbiota diversity in healthy individuals in China. *BMC Infectious Diseases*, 18(1), 207. doi:10.1186/s12879-018-3111-z

Dou, S., Gadonna-Widehem, P., Rome, V., Hamoudi, D., Rhazi, L., Lakhal, L., . . . Abdennebi-Najar, L. (2017). Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS One*, 12(1), e0169851. doi:10.1371/journal.pone.0169851

Douglas, B., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1-48. doi:10.18637/jss.v067.i01

Doyle, M. E. (2001, March). Alternatives to antibiotic use for growth promotion in animal husbandry. Retrieved from https://www.iatp.org/documents/alternatives-to-antibiotic-use-for-growth-promotion-in-animal-husbandry

Dunne, H. W. (1959). An expanding concept of colibacillosis with emphasis on the disease in swine. *Canadian Jornal of Comprative Medicine*, 23(3), 101-104.

EcL. (2004). Pathogenic *E. coli*. Pathogenesis. Retrieved from http://www.ecl-lab.ca/en/ecoli/pathogenesis.asp

Edmonds, M. S., Izquierdo, O. A., & Baker, D. H. (1985). Feed additive studies with newly weaned pigs: efficacy of supplemental copper, antibiotics and organic acids. *Journal of Animal Science*, 60(2), 462-469. doi:10.2527/jas1985.602462x

EFSA, & ECDC. (2017). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015. *EFSA Journal*, 15(2), 4694. doi:10.2903/j.efsa.2017.4694

EFSA. (2018). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. *EFSA Journal*, 16(2), 5182. doi:10.2903/j.efsa.2018.5182

Eisemann, J. H., & van Heugten, E. (2007). Response of pigs to dietary inclusion of formic acid and ammonium formate. *Journal of Animal Science*, 85(6), 1530-1539. doi:10.2527/jas.2006-464

Emebet, D., Awoke, K., & Asenakew, A. (2017). Challenges, opportunities and management practice of pig production in Debre Markos Town, West Goijam Zone in Amahra Regional State, Ethiopia. *Poultry, Fisheries and Wildlife Sciences*, 5, 176. doi:10.4172/2375-446X.1000176

Ettle, T., Mentschel, K., & Roth, F. X. (2004). Dietary self-selection for organic acids by the piglet. *Archives of Animal Nutrition*, 58(5), 379-388. doi:10.1080/00039420400005067

Evans Jr, D. J., & Evans, D. G. (1996). *Escherichia coli* in diarrheal disease. In S. Baron (Ed.), *Medical Mmicrobiology* (4th ed.). Galveston, TX: The University of Texas Medical Branch at Galveston.

Fàbrega, A., & Vila, J. (2013). *Salmonella enterica* serovar Typhimurium skills to succeed in the host: Virulence and regulation. *Clinical Microbiology Reviews*, 26(2), 308-341. doi:10.1128/CMR.00066-12

Fairbrother, J. M., Nadeau, E., & Gyles, C. L. (2005). *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews*, 6(1), 17–39. doi:10.1079/AHR2005105

Fairbrother, J. M., & Gyles, C. L. (2012). Colibacillosis. In J. J. Zimmerman, L. A. Karriker, A. Ramirez, K. J. Schwartz, & G. W. Stevenson (Eds.), *Diseases of swine* (10th ed., pp. 821-833). Chichester, UK: Wiley-Blackwell.

Fang, C. L., Sun, H., Wu, J., Niu, H. H., & Feng, J. (2014). Effects of sodium butyrate on growth performance, haematological and immunological characteristics of weanling piglets. *Journal of Animal Physiology and Animal Nutrition*, 98(4), 680-685. doi:10.1111/jpn.12122

Feng, W., Wu, Y., Chen, G., Fu, S., Li, B., Huang, B., . . . Liu, J. (2018). Sodium butyrate attenuates diarrhea in weaned piglets and promotes tight junction protein expression in colon in a GPR109A-dependent manner. *Cellular Physiology and Biochemistry*, 47(4), 1617-1629. doi:10.1159/000490981

Ferrara, F., Tedin, L., Pieper, R., Meyer, W., & Zentek, J. (2017). Influence of medium-chain fatty acids and short-chain organic acids on jejunal morphology and intra-epithelial immune cells in weaned piglets. *Journal of Animal Physiology and Animal Nutrition*, 101(3), 531-540. doi:10.1111/jpn.12490

Foster, J. W. (2004). *Escherichia coli* acid resistance: Tales of an amateur acidophile. *Nature Reviews. Microbiology*, 2(11), 898-907. doi:10.1038/nrmicro1021

Foster, K. R., Schluter, J., Coyte, K. Z., & Rakoff-Nahoum, S. (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nature*, 548, 43-51. doi:10.1038/nature23292

Franco, L. D., Fondevila, M., Lobera, M. B., & Castrillo, C. (2005). Effect of combinations of organic acids in weaned pig diets on microbial species of digestive tract contents and their response on digestibility. *Journal of Animal Physiology and Animal Nutrition*, 89(3-6), 88-93. doi:10.1111/j.1439-0396.2005.00553.x

Frese, S. A., Parker, K., Calvert, C. C., & Mills, D. A. (2015). Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome*, 3, 28. doi:10.1186/s40168-015-0091-8

Gaggìa, F., Mattarelli, P., & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology*, 141(S1), S15-S28. doi:10.1016/j.ijfoodmicro.2010.02.031

Gálfi, P., & Bokori, J. (1990). Feeding trial in pigs with a diet containing sodium n-butyrate. *Acta Veterinaria Hungarica*, 38(1-2), 3-17.

Gallois, M., Gidenne, T., Orengo, J., Caubet, C., Tasca, C., Milon, A., & Boullier, S. (2008). Testing the efficacy of medium chain fatty acids against rabbit colibacillosis. *Veterinary Microbiology*, 131(1-2), 192-198. doi:10.1016/j.vetmic.2008.03.004

Gaudier, E., Jarry, A., Blottière, H. M., Coppet, P., Buisine, M. P., Aubert, J. P., . . . Hoebler, C. (2004). Butyrate specifically modulates *MUC* gene expression in intestinal epithelial goblet cells deprived of glucose. American Journal of Physiology. *Gastrointestinal and Liver Physiology*, 287(6), G1168-G1174. doi:10.1152/ajpgi.00219.2004

Gauthier, R. (2018, December). To protect or not to protect? That is the question! Retrieved from https://www.pigprogress.net/Special-Focus/Alternative-Growth-Promotion/To-protect-or-not-to-protect-That-is-the-question/

Gebru, E., Lee, J. S., Son, J. C., Yang, S. Y., Shin, S. A., Kim, B., . . . Park, S. C. (2010). Effect of probiotic-, bacteriophage-, or organic acid-supplemented feeds or fermented soybean meal on the growth performance, acute-phase response, and bacterial shedding of grower pigs challenged with *Salmonella enterica* serotype Typhimurium. *Journal of Animal Science*, 88(12), 3880-3886. doi:10.2527/jas.2010-2939

Geng, C. Y., Zhang, M., Li, C. Y., Jin, Y. H., Yan, C. G., & Jin, Y. (2016a). Effectiveness of medium-chain fatty acids on feed intake and weight gain in animal: Depending on balance. *Indian Journal of Animal Research*, 50(6), 885-892. doi:10.18805/ijar.v0iOF.4554

Geng, S., Zhu, W., Xie, X., Li, X., Wu, J., Liang, Z., . . . Zhong, C. (2016b). Medium-chain triglyceride ameliorates insulin resistance and inflammation in high fat diet-induced obese mice. *European Journal of Nutrition*, 55(3), 931-940. doi:10.1007/s00394-015-0907-0

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition*, 125(6), 1401-1412. doi:10.1093/jn/125.6.1401

Giesting, D. W., & Easter, R. A. (1986). Acidification. Status in swine diets. *Feed Management*, 37, 8-10.

Gilbert, M. S., Ijssennagger, N., Kies, A. K., & van Mil, S. W. C. (2018). Protein fermentation in the gut; implications for the intestinal dysfunction in humans, pigs and poultry. *American Journal of Physiology. Gastrointestinal* and Liver Phyisiology, 315, G159-G170. doi:10.1152/ajpgi.00319.2017

Gill, C., van den Wijgert, J. H. H. M., Blow, F., & Darby, A. C. (2016). Evaluation of lysis methods for the extraction of bacterial DNA for analysis of the vaginal microbiota. *PLoS One*, 11(9), e0163148. doi:10.1371/journal.pone.0163148

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., & Egozcue, J. J. (2017). Microbiome datasets are compositional: and this is not optional. *Frontiers in Microbiology*, 8, 2224. doi:10.3389/fmicb.2017.02224

Gomez-Arango, L. F., Barrett, H. L., Wilkinson, S. A., Callaway, L. K., McIntyre, H. D., Morrison, M., & Dekker Nitert, M. (2018). Low dietary fiber intake increases *Collinsella* abundance in the gut microbiota of overweight and obese pregnant women. *Gut Microbes*, 9(3), 189-201. doi:10.1080/19490976.2017.1406584

Gonzalez, L. M., Moeser, A. J., & Blikslager, A. T. (2015). Porcine models of digestive disease: The future of large animal translational research. *Translational Research*, 166(1), 12-27. doi:10.1016/j.trsl.2015.01.004

González-Ramón, N., Alava, M. A., Sarsa, J. A., Piñeiro, M., Escartin, A., Garcia-Gil, A., . . . Piñeiro, A. (1995). The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120. *FEBS Letters*, 371(3), 227-230. doi:10.1016/0014-5793(95)00882-A

Gresse, R., Chaucheyras-Durand, F., Fleury, M. A., Van de Wiele, T., Forano, E., Blanquet-Diot, S. (2017). Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to Health. *Trends in Microbiology*, 25(10). 851-873. doi:10.1016/j.tim.2017.05.004

Grilli, E., Vitari, F., Domeneghini, C., Palmonari, A., Tosi, G., Fantinati, P., . . . Piva, A. (2013). Development of a feed additive to reduce caecal *Campylobacter jejuni* in broilers at slaughter age: from *in vitro* to *in vivo*, a proof of concept. *Journal of Applied Microbiology*, 114(2), 308-317. doi:10.1111/jam.12053

Grimont, P. A., & Weill, F. X. (2007). Antigenic formulae of the *Salmonella* serovars. *World Health Organization Technical Report Series*. doi:10.1038/nature12153

Guerra-Ordaz, A. A., González-Ortiz, G., La Ragione, R. M., Woodward, M. J., Collins, J. W., Pérez, J. F., & Martín-Orúe, S. M. (2014). Lactulose and *Lactobacillus plantarum*, a potential

complementary synbiotic to control postweaning colibacillosis in piglets. *Applied and Environmental Microbiology*, 80(16), 4879-4886. doi:10.1128/AEM.00770-14

Guevarra, R. B., Hong, S. H., Cho, J. H., Kim, B. R., Shin, J., Lee, J. H., . . . Kim, H. B. (2018). The dynamics of the piglet gut microbiome during the weaning transition in association with health and nutrition. *Journal of Animal Science and Biotechnology*, 9, 54. doi:10.1186/s40104-018-0269-6

Guillot, E., Vaugelade, P., Lemarchal, P., & Rérat, A. (1993). Intestinal absoprtion and liver uptake of medium-chain fatty acids in non-anaesthetized pigs. *British Journal of Nutrition*, 69(2), 431-442. doi:10.1079/BJN19930045

Gunsalus, K. T., Tornberg-Belanger, S. N., Matthan, N. R., Lichtenstein, A. H., & Kumamoto, C. A. (2016). Manipulation of host diet to reduce gastrointestinal colonization by the opportunistic pathogen *Candida albicans*. *mSphere*, 1(1), e00020-15. doi:10.1128/mSphere.00020-15

Gustavo Hermes, R., Molist, F., Pérez, J. F., Gómez de Segura, A., Ywazaki, M., Davin, R., . . . Martín-Orúe, S. M. (2013). Casein glycomacropeptide in the diet may reduce *Escherichia coli* attachment to the intestinal mucosa and increase the intestinal lactobacilli of early weaned piglets after an enterotoxigenic *E. coli* K88 challenge. *British Journal of Nutrition*, 109(6), 1001-1012. doi:10.1017/S0007114512002978

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., . . . Birren, B. W. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Research*, 21(3), 494-504. doi:10.1101/gr.112730.110

Halas, D., Heo, J. M., Hansen, C. F., Kim, J. C., Hampson, D. J., Mullan, B. P., & Pluske, P. R. (2007). Organic acids, prebiotics and protein level as dietary tools to control the weaning transition and reduce post-weaning diarrhoea in piglets. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 2, 079. doi:10.1079/PAVSNNR20072079

Hampson, D. J. (1986). Alterations in piglet small intestinal structure at weaning. *Research in Veterinary Science*, 40(1), 32-40. doi:10.1016/S0034-5288(18)30482-X

Han, Y. K., Hwan, I. L. H., & Thacker, P. A. (2011). Use of a micro-encapsulated eucalyptus-medium chain fatty acid product as an alternative to zinc oxide and antibiotics for weaned pigs. *Journal of Swine Health and Production*, 19(1), 34-43.

Han, G. G., Lee, J. Y., Jin, G. D., Park, J., Choi, Y. H., Kang, S. K., . . . Choi, Y. (2018). Tracing of the fecal microbiota of commercial pigs at five growth stages from birth to shipment. *Scientific Reports*, 8(1), 6012. doi:10.1038/s41598-018-24508-7

Hanczakowska, E., Świątkiewicz, M., Hanczakowski, P., & Wróbel, A. (2010). Medium-chain fatty acids as feed supplements for weaned piglets. *Medycyna Weterynaryjna*, 66(5), 331-334.

Hanczakowska, E., Szewczyk, A., & Okoń, K. (2011). Effects of dietary caprylic and capric acids on piglet performance and mucosal epithelium structure of the ileum. *Journal of Animal and Feed Sciences*, 20(4), 556-565. doi:10.22358/jafs/66213/2011

Hanczakowska, E., Szewczyk, A., Świątkiewicz, M., & Okoń, K. (2013). Short- and medium-chain fatty acids as a feed supplement for weaning and nursery pigs. *Polish Journal of Veterinary Sciences*, 16(4), 647-654. doi:10.2478/pjvs-2013-0092

Hanczakowska, E., Niwińska, B., Grela, E. R., Węglarzy, K., & Okoń, K. (2016a). The effect of supplementing sodium butyrate containing feed with glutamine and/or glucose on the structure of the piglet digestive tract and selected blood indices. *Annals of Animal Science*, 16(4), 1127-1139. doi:10.1515/aoas-2016-0039

Hanczakowska, E., Świątkiewicz, M., Natonek-Wiśniewska, M., & Okoń, K. (2016b). Medium chain fatty acids (MCFA) and/or probiotic *Enterococcus faecium* as a feed supplement for piglets. *Livestock Science*, 192, 1-7. doi:10.1016/j.livsci.2016.08.002

Hanczakowska, E. (2017). The use of medium chain fatty acids in piglet feeding – a review. *Annals of Animal Science*, 17(4), 967-977. doi:10.1515/aoas-2016-0099

Hansen, C. F., Riis, A. L., Bresson, S., Højbjerg, O., & Jensen, B. B. (2007). Feeding organic acids enhances the barrier function against pathogenic bacteria of the piglet stomach. *Livestock Science*, 108(1-3), 206-209. doi:10.1016/j.livsci.2007.01.059

Harada, E., Niiyama, M., & Syuto, B. (1986). Comparison of pancreatic exocrine secretion via endogenous secretin by intestinal infusion of hydrochloric acid and monocarboxylic acid in anesthetized piglets. *The Japanese Journal of Physiology*, 36(5), 843-856.

He, B., Wang, M., Guo, H., Jia, Y., Yang, X., & Zhao, R. (2016). Effects of sodium butyrate supplementation on reproductive performance and colostrum composition in gilts. *Animal*, 10(10), 1722-1727. doi:10.1017/S1751731116000537

Heinritz, S. N., Weiss, E., Eklund, M., Aumiller, T., Louis, S., Rings, A., . . . Mosenthin, R. (2016). Intestinal microbiota and microbial metabolites are changed in a pig model fed a high-fat/low-fiber or a low-fat/high-fiber diet. *PLoS One*, 11(4), e0154329. doi:10.1371/journal.pone.0154329

Heo, J. M., Opapeju, F. O., Pluske, J. R., Kim, J. C., Hampson, D. J., & Nyachoti, C. M. (2013). Gastrointestinal health and function in weaned pigs: A review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *Journal of Animal Physiology and Animal Nutrition*, 97(2), 207-237. doi:10.1111/j.1439-0396.2012.01284.x

Heppner, K. M., Chaudhary, N., Müller, T. D., Kirchner, H., Habegger, K. M., Ottaway, N., . . . Tschöp, M. H. (2012). Acylation type determines ghrelin's effects on energy homeostasis in rodents. *Endocrinology*, 153(10), 4687-4695. doi:10.1210/en.2012-1194

Hernández, A., & Pluske, J. (2008). Using dietary medium-chain triglycerides to improve post-weaning performance of pigs. CRC 2B-102/103-0506, pp. 1-13.

HMP. (2019). National Institutes of Health (NIH). Human Microbiome Project (HMP). Retrieved from https://hmpdacc.org/

Holman, D. B., Brunelle, B. W., Trachsel, J., & Allen, H. K. (2017). Meta-analysis to define a core microbiota in the swine gut. *mSystems*, 2(3), e00004-17. doi:10.1128/mSystems.00004-17

Hong, S. M., Hwang, J. H., & Kim, I. H. (2012). Effect of medium-chain trygliceride (MCT) on growth performance, nutrient digestibility, blood characteristics in weanling pigs. *Asian-Australasian Journal of Animal Science*, 25(7), 1003-1008. doi:10.5713/ajas.2011.11402

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, 50(3), 346-363. doi:10.1002/bimj.200810425

Hou, Y., Wang, L., Yi, D., Ding, B., Chen, X., Wang, Q., . . . Wu, G. (2014). Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. *British Journal of Nutrition*, 111(10), 1748-1458. doi:10.1017/S0007114514000038

Hoytema van Konijnenburg, D. P., Reis, B. S., Pedicord, V. A., Farache, J., Victora, G. D., & Mucida, D. (2017). Intestinal epithelial and intraepithelial T cell crosstalk mediates a dynamic response to infection. *Cell*, 171(4), 783-794. doi:10.1016/j.cell.2017.08.046

Htoo, J. K., & Molares, J. (2012). Effects of dietary supplementation with two potassium formate sources on performance of 8- to 22-kg pigs. *Journal of Animal Science*, 90(S4), 346-349. doi:10.2527/jas.53776

Huang, C., Song, P., Fan, P., Hou, C., Thacker, P., & Ma, X. (2015). Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. *The Journal of Nutrition*, 145(12), 2774-2780. doi:10.3945/jn.115.217406

Hunt, J. N., & Knox, M. T. (1968). A relation between the chain length of fatty acids and the slowing of gastric emptying. *The Journal of Physiology*, 194(2), 327-336.

Hunt, J. N., & Knox, M. T. (1969). The slowing of gastric emptying by nine acids. *The Journal of Physiology*, 20(1), 161-179. doi:10.1113/jphysiol.1972.sp009793

Imran, S., Gillis, R. B., Kok, M. S., Harding, S. E., & Adams, G. G. (2012). Application and use of inulin as a tool for therapeutic drug delivery. *Biotechnology and Genetic Engineering Reviews*, 28, 33-45. doi:10.5661/bger-28-33

Ingerslev, A. K., Mutt, S. J., Lrke, H. N., Hedemann, M. S., Theil, P. K., Nielsen, K. L., . . . Knudsen, K. E. (2017). Postprandial PYY increase by resistant starch supplementation is independent of net portal appearance of short-chain fatty acids in pigs. *PLoS One*, 12(10), e0185927. doi:10.1371/journal.pone.0185927

Iyer, M. N., Sarmah, B. C., Tamuli, M. K., Das, A., & Kalita, D. (2012). Effect of dietary sunflower oil and coconut oil on adipose tissue gene expression, fatty acid composition and serum lipid profile of grower pigs. *Archives of Animal Nutrition*, 66(4), 271-282. doi:10.1080/1745039X.2012.683324

Jambor de Sousa, U. L., Benthem, L., Arsenijevic, D., Scheurink, A. J., Langhans, W., Geary, N., & Leonhardt, M. (2006). Hepatic-portal oleic acid inhibits feeding more potently than hepatic-portal caprylic acid in rats. *Physiology and Behavior*, 89(3), 329-334. doi:10.1016/j.physbeh.2006.06.020

Jensen, P., & Recén, B. (1989). When to wean - Observations from free-ranging domestic pigs. *Applied Animal Behavior Science*, 23(1-2), 49-60. doi:10.1016/0168-1591(89)90006-3

Jensen, M. T., Cox, R. P., & Jensen, B. B. (1995). Microbial production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat. *Animal Science*, 61(2), 293-304. doi:10.1017/S1357729800013837

Jensen, B. B. (1998). The impact of feed additives on the microbial ecology of the gut in young pigs. *Journal of Animal and Feed Sciences*, 7 (S1), 45-64. doi:10.22358/jafs/69955/1998

Jensen, G. M., Frydendahl, K., Svendsen, O., Jørgensen, C. B., Cirera, S., Fredholm, M., . . . Møller, K. (2006). Experimental infection with *Escherichia coli* O149:F4ac in weaned piglets. *Veterinary Microbiology*, 115(1-3), 243-249. doi:10.1016/j.vetmic.2006.01.002

Jiang, Y., Zhang, W., Gao, F., & Zhou, G. (2015). Effect of sodium butyrate on intestinal inflammatory response to lipopolysaccharide in broiler chickens. *Canadian Journal of Animal Science*, 95(3), 389-395. doi:10.4141/CJAS-2014-183

Jiao, A. R., Diao, H., Yu, B., He, J., Yu, J., Zheng, P., . . . Chen, D. W. (2018). Oral administration of short chain fatty acids could attenuate fat deposition of pigs. *PLoS One*, 13(5), e0196867. doi:10.1371/journal.pone.0196867

Jiminez, J. A., Uwiera, T. C., Douglas Inglis, G., & Uwiera, R. R. (2015). Animal models to study acute and chronic intestinal inflammation in mammals. *Gut Pathogens*, 7, 29. doi:10.1186/s13099-015-0076-y

Jondreville, C., Revy, P. S., & Dourmad, J. Y. (2003). Dietary means to better control the environmental impact of copper and zinc by pigs from weaning to slaughter. *Livestock Production Science*, 84(2), 147-156. doi:10.1016/j.livprodsci.2003.09.011

Jong, A. J., Kloppenburg, M., Toes, R. E. M., & Ioan-Facsinay, A. (2014). Fatty acids, lipid mediators, and T-cell function. *Frontiers in Immunology*, 5, 483. doi:10.3389/fimmu.2014.00483

Kanno, M., Katayama, T., Morita, N., Tamaki, H., Hanada, S., & Kamagata, Y. (2015). *Catenisphaera adipataccumulans* gen. nov., sp. Nov., a membre of the family Erysipelotrichaceae isolated from an anaerobic digester. *International Journal of Systematic and Evolutionary Microbiology*, 65, 805-810. doi:10.1099/ijs.0.000021

Khanvilkar, A. M., Ranveer, R. C., & Sahoo, A. K. (2016). Carrier materials for encapsulation of bio-active components of food. *International Journal of Pharmaceutical Sciences Review and Research*, 40(1), 62-73.

Kien, C. L., Blauwiekel, R., Bunn, J. Y., Jetton, T. L., Frankel, W. L., & Holst, J. J. (2007). Cecal infusion of butyrate increases intestinal cell proliferation in piglets. *The Journal of Nutrition*, 137(4), 916-922. doi:10.1093/jn/137.4.916

Kil, D. Y., Piao, L. G., Long, H. F., Lim, J. S., Yun, M. S., Kong, C. S., . . . Kim, Y. Y. (2006). Effects of organic or inorganic acid supplementation on growth performance, nutrient digestibility and white blood cell counts in weanling pigs. *Asian-Australasian Journal of Animal Sciences*, 19(2), 252-261. doi:10.5713/ajas.2006.252

Kim, H. B., Borewicz, K., White, B. A., Singer, R. S., Sreevatsan, S., Tu, Z. J., & Isaacson, R. E. (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veterinary Microbiology*, 153(1-2), 24-133. doi:10.1016/j.vetmic.2011.05.021

Kim, H. B., & Isaacson, R. E. (2015). The pig gut microbial diversity: Understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Veterinary Microbiology*, 177(3-4), 242-251. doi:10.1016/j.vetmic.2015.03.014

Kogut, M. H., & Arsenault, R. J. (2016). Editorial: Gut health: The new paradigm in food animal production. Frontiers in Veterinary Science, 3, 71. doi:10.3389/fvets.2016.00071

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., & Bäckhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell*, 165(65), 1332-1345. doi:10.1016/j.cell.2016.05.041

Kono, H., Fujii, H., Asakawa, M., Maki, A., Amemiya, H., Hirai, Y., . . . Yamamoto, M. (2004). Medium-chain triglycerides enhance secretory IgA expression in rat intestine after administration of endotoxin. *American Journal of Physiology. Gastrointestinal* and Liver *Physiology*, 286(6), G1081-G1089. doi:10.1152/ajpgi.00457.2003

Kranker, S., Alban, L., Boes, J., & Dahl, J. (2003). Longitudinal study of *Salmonella enterica* serotype Typhimurium infection in three Danish farrow-to-finish swine herds. *Journal of Clinical Microbiology*, 41(6), 2282-2288. doi:10.1128/JCM.41.6.2282-2288.2003

Krzyściak, W., Pluskwa, K. K., Jurczak, A., & Kościelniak, D. (2013). The pathogenicity of the *Streptococcus* genus. *European Journal of Clinical Microbiology and Infectious* Diseases, 32(11), 1361-1376. doi:10.1007/s10096-013-1914-9

Kuang, Y., Wang, Y., Zhang, Y., Song, Y., Zhang, X., Lin, Y., . . . Fang, Z. (2015). Effects of dietary combinations of organic acids and medium chain fatty acids as a replacement of zinc oxide on growth, digestibility and immunity of weaned pigs. *Animal Feed and Science Technology*, 208, 145-157. doi:10.1016/j.anifeedsci.2015.07.010

Lacerda, E. C. W., Calado, V. M. A., Monteiro, M., Finotelli, P. V., Torres, A. G., & Perrone, D. (2016). Starch, inulin and maltodextrin as encapsulating agents affect the quality and stability of jussara pulp microparticles. *Carbohydrate Polymers*, 151, 500-510. doi:10.1016/j.carbpol.2016.05.093

Lacorn, M., Goerke, M., & Claus, R. (2010). Inulin-coated butyrate increases ileal *MCT1* expression and affects mucosal morphology in the porcine ileum by reduced apoptosis. *Journal of Animal Physiology and Animal Nutrition*, 94(5), 670-676. doi:10.1111/j.1439-0396.2009.00955.x

Lai, W. K., Yen, H. C., Lin, C. S., & Chiang, S. H. (2014). The effects of dietary medium-chain triacylglycerols on growth performance and intestinal microflora in young pigs. *Journal of Animal and Feed Sciences*, 23(4), 331-336. doi:10.22358/jafs/65669/2014

Lallès, J. P., Bosi, P., Smidt, H., & Stokes, C. R. (2007). Nutritional management of gut health in pigs around weaning. *The Proceedings of the Nutrition Society*, 66(2), 260-268. doi:10.1017/S0029665107005484

Lamendella, R., Domingo, J. W., Ghosh, S., Martinson, J., & Oerther, D. B. (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiology*, 11, 103. doi:10.1186/1471-2180-11-103

Larraufie, P., Martin-Gallausiaux, C., Lapaque, N., Doré, J., Gribble, F. M., Reimann, F., & Blottière, H. M. (2018). SCFAs strongly stimulate PYY production in human enteroendocrine cells. *Scientific Reports*, 8, 74. doi:10.1038/s41598-017-18259-0

Laudadio, I., Fulci, V., Palone, F., Stronati, L., Cucchiara, S., & Carissimi, C. (2018). Quantitative assessment of shotgun metagenomics and 16S rDNA amplicon sequencing in the study of human gut microbiome. *OMICS: A Journal of Integrative Biology*, 22(4), 248-254. doi:10.1089/omi.2018.0013

Lawlor, P. G., Lynch, P. B., Caffrey, P. J., O'Reilly, J. J., & O'Connell, M. K. (2005). Measurements of the acid-binding capacity used in pig diets. *Irish Veterinary Journal*, 58(8), 447-452. doi:10.1186/2046-0481-58-8-447

Le Gall, M., Gallois, M., Sève, B., Louveau, I., Holst, J. J., Oswald, I. P., . . . Guilloteau, P. (2009). Comparative effect of orally administered sodium butyrate before or after weaning on growth and several indices of gastrointestinal biology of piglets. *British Journal of Nutrition*, 102(9), 1285-1296. doi:10.1017/S0007114509990213

Leatham, M. P., Banerjee, S., Autieri, S. M., Mercado-Lubo, R., Conway, T., & Cohen, P. S. (2009). Precolonized human commensal *Escherichia coli* strains serve as a barrier to *E. coli* O157:H7 growth in the streptomycin-treated mouse intestine. *Infection and Immunity*, 77(7), 2876-2886. doi:10.1128/IAI.00059-09

Lee, H. F., & Chiang, S. H. (1994). Energy value of medium-chain triglycerides and their efficacy in improving survival. *Journal of Animal Science*, 72(1), 133-138.

Lee, S. I., & Kang, K. S. (2017). Function of capric acid in cyclophosphamide-induced intestinal inflammation, oxidative stress, and barrier function in pigs. *Scientific Reports*, 7, 16530. doi:10.1038/s41598-017-16561-5

Lee, J., Taip, F. S., & Abdullah, Z. (2018). Effectiveness of additives in spray drying performance: a review. *Food Research*, 2(6), 486-499. doi:10.26656/fr.2017.2(6).134

Lei, X. J., Park, J. W., Baek, D. H., Kim, J. K., & Kim, I. H. (2017). Feeding the blend of organic acids and medium chain fatty acids reduces the diarrhea in piglets orally challenged with enterotoxigenic *Escherichia coli* K88. *Animal Feed Science and Technology*, 224, 46-51. doi:10.1016/j.anifeedsci.2016.11.016

Leite, F. L. L., Singer, R. S., Ward, T., Gebhart, C. J., & Isaacson, R. E. (2018). Vaccination against Lawsonia intracellularis decreases shedding of *Salmonella enterica* serovar Typhimurium in co-infected pigs and alters the gut microbiome. *Scientific Reports*, 8, 2857. doi:10.1038/s41598-018-21255-7

Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., & Møller, K. (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology*, 68(2), 673-690. doi:10.1128/AEM.68.2.673-690.2002

Lewis, K., Lutgendorff, F., Phan, V., Söderholm, J. D., Sherman, P. M., & McKay, D. M. (2010). Enhanced translocation of bacteria across metabolically stressed epithelia is reduced by butyrate. *Inflammatory Bowel Diseases*, 16(7), 1138-1148. doi:10.1002/ibd.21177

Li, Z., Yi, G., Yin, J., Sun, P., Li, D., & Knight, C. (2008). Effects of organic acids on growth performance, gastrointestinal pH, intestinal microbial populations and immune responses of weaned pigs. *Asian-Australasian Journal of Animal Sciences*, 21(2), 252-261. doi:10.5713/ajas.2008.70089

Li, Y., Zhang, H., Yang, L., Zhang, L., & Wang, T. (2015). Effect of medium-chain triglycerides on growth performance, nutrient digestibility, plasma metabolites and antioxidant capacity in weanling pigs. *Animal Nutrition*, 1(1), 12-18. doi:10.1016/j.aninu.2015.02.001

Li, Z., Wang, W., Liu, D., & Guo, Y. (2017). Effects of *Lactobacillus acidophilus* on gut microbiota composition in broilers challenged with *Clostridium perfringens*. *PLoS One*, 12(11), e0188634. doi:10.1371/journal.pone.0188634

Li, S., Zheng, J., Deng, K., Chen, L., Zhao, X. L., Jiang, X., . . . Wu, D. (2018). Supplementation with organic acids showing different effects on growth performance, gut morphology and microbiota of weaned pigs fed with highly or less digestible diets. *Journal of Animal Science*, 96(8), 3302-3318. doi:10.1093/jas/sky197

Lim, M. Y., Song, E. J., Kim, S. H., Lee, J., & Nam, Y. D. (2018). Comparison of DNA extraction methods for human gut microbial community profiling. *Systematic and Applied Microbiology*, 41(2), 151-157. doi:10.1016/j.syapm.2017.11.008

Lima, T. M., Kanunfre, C. C., Pompéia, C., Verlengia, R., & Curi, R. (2002). Ranking the toxicity of fatty acids on Jurkat and Raji cells by flow cytometric analysis. *Toxicology in Vitro*, 16(6), 741-747.

Liu, C., Yan, S., Liu, W., Wang, R., Wang, J., & Liu, W. (2010). Preparation and characterization of medium-chain fatty acid liposomes by lyophilization. *Journal of Liposome Research*, 20(3), 183-190. doi:10.3109/08982100903244500

Liu, Y. (2015). Fatty acids, inflammation and intestinal health in pigs. *Journal of Animal Science and Biotechnology*, 6(1), 41. doi:10.1186/s40104-015-0040-1

Liu, Y., Espinosa, C. D., Abelilla, J. J., Casas, G. A., Lagos, L. V., Lee, S. A., . . . Stein, H. H. (2018). Non-antibiotic feed additives in diets for pigs: A review. *Animal Nutrition*, 4(2), 113-125. doi:10.1016/j.aninu.2018.01.007

Long, S. F., Xu, Y. T., Pan, L., Wang, Q. Q., Wang, C. L., Wu, J. Y., . . . Piao, X. S. (2018). Mixed organic acids as antibiotic substitutes improve performance, serum immunity, intestinal morphology and microbiota for weaned piglets. *Animal Feed Science and Technology*, 235, 23-32. doi:10.1016/j.anifeedsci.2017.08.018

Looft, T., Allen, H. K., Cantarel, B. L., Levine, U. Y., Bayles, D. O., Alt, D. P., . . . Stanton, T. B. (2014a). Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *The ISME Journal*, 8(8), 1566-1576. doi:10.1038/ismej.2014.12

Looft, T., Allen, H. K., Casey, T. A., Alt, D. P., & Stanton, T. B. (2014b). Carbadox has both temporary and lasting effects on the swine gut microbiota. *Frontiers in Microbiology*, 5, 276. doi:10.3389/fmicb.2014.00276

Loynachan, A. T. (2005). *Acute Salmonella infection in swine* (Ph.D. dissertation). Retrieved from Iowa State University Digital Repository. (Accession No. 3190722)

Lu, J. J., Zou, X. T., & Wang, Y. M. (2008). Effects of sodium butyrate on the growth performance, intestinal microfl ora and morphology of weanling pigs. *Journal of Animal and Feed Sciences*, 17(4), 568-578. doi:10.22358/jafs/66685/2008

Lu, H., Su, S., & Ajuwon, K. M. (2012). Butyrate supplementation to gestating sows and piglets induces muscle and adipose tissue oxidative genes and improves growth performance. *Journal of Animal Science*, 90(S4), 430-432. doi:10.2527/jas.53817

Lückstädt, C., & Mellor, S. (2011). The use of organic acids in animal nutrition, with special focus on dietary potassium diformate under European and Austral-Asian conditions. *Recent Advances in Animal Nutrition* – Australia, 18, 123-130.

Luise, D., Motta, V., Salvarani, C., Chiappelli, M., Fusco, L., Bertocchi, M., . . . Trevisi, P. (2017). Long-term administration of formic acid to weaners: Influence on intestinal microbiota, immunity parameters and growth performance. *Animal Feed Science and Technology*, 232, 160-168. doi:10.1016/j.anifeedsci.2017.06.015

Lustri, B. C., Sperandio, V., & Moreira, C. G. (2017). Bacterial chat: Intestinal metabolites and signals in host-microbiota-pathogen interactions. *Infection and Immunity*, 85(12), e00476-17. doi:10.1128/IAI.00476-17

Lutwick, L. I. (2014). Enterohemorrhagic *E. coli* infections. In A. Yamada, L. H. Kahn, B. Kaplan, T. P. Monath, J. Woodall, & L. Conti (Eds.), *Confronting emerging zoonoses* (pp. 77-111). Springer. doi:10.1007/978-4-431-55120-1_5

Lynch, M. D. J., & Neufeld, J. D. (2015). Ecology and exploration of the rare biosphere. *Nature*, 13, 217-229. doi:10.1038/nrmicro3400

Macfarlane, G. T., & Macfarlane, S. (1997). Human colonic microbiota: Ecology, physiology and metabolic potential of intestinal bacteria. *Scandinavian Journal of Gastroenterology*, 222, S3-S9. doi:10.1080/00365521.1997.11720708

Mach, N., Berri, M., Estellé, J., Levenez, F., Lemonnier, G., Denis, C., . . . Lepage, P. (2015). Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environmental Microbiology Reports*, 7(3), 554-569. doi:10.1111/1758-2229.12285

Madene, A., Jacquot, M., Scher, J., & Desobry, S. (2006). Flavour encapsulation and controlled release - A review. *International Journal of Food Science and Technology*, 41(1), 1-21. doi:10.1111/j.1365-2621.2005.00980.x

Mailhe, M., Ricaboni, D., Vitton, V., Gonalez, J. M., Bachar, D., Dubourg, G., . . . Raoult, D. (2018). Repertoire of the gut microbiota from stomach to colon using culturomics and next-generation sequencing. *BMC Microbiology*, 18, 157. doi:10.1186/s12866-018-1304-7

Mallick, H., Ma, S., Franzosa, E. A., Vatanen, T., Morgan, X. C., & Huttenhower, C. (2017). Experimental design and quantitative analysis of microbial community multiomics. *Genome Biology*, 18, 228. doi:10.1186/s13059-017-1359-z

Mallo, J. J., Balfagón, A., Gracia, M. I., Honrubia, P., & Puyalto, M. (2012). Evaluation of different protections of butyric acid aiming for release in the last part of the gastrointestinal tract of piglets. *Journal of Animal Science*, 90(S4), 227-229. doi:10.2527/jas.53959

Mann, E., Schmitz-Esser, S., Zebeli, Q., Wagner, M., Ritzmann, M., & Metzler-Zebeli, B. U. (2014). Mucosa-associated bacterial microbiome of the gastrointestinal tract of weaned pigs and dynamics linked to dietary calcium-phosphorus. *PLoS One*, 9(1), e86950. doi:10.1371/journal.pone.0086950

Manohar, V., Echard, B., Perricone, N., Ingram, C., Enig, M., Bagchi, D., & Preuss, H. G. (2013). *In vitro* and *in vivo* effects of two coconut oils in comparison to monolaurin on *Staphylococcus aureus*: Rodent studies. *Journal of Medicinal Food*, 16(6), 499-503. doi:10.1089/jmf.2012.0066

Manzanilla, E. G., Pérez, J. F., Martin, M., Kamel, C., Baucells, F., & Gasa, J. (2004). Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. *Journal of Animal Science*, 82(11), 3210-3218. doi:10.2527/2004.82113210x

Manzanilla, E. G., Nofrarías, M., Anguita, M., Castillo, M., Pérez, J. F., Martín-Orúe, S. M., . . . Gasa, J. (2006). Effects of butyrate, avilamycin, and a plant extract combination on the intestinal equilibrium of early-weaned pigs. *Journal of Animal Science*, 84(10), 2743-2751. doi:10.2527/jas.2005-509

Manzke, N. E., Gomes, B. K., Xavier, E. G., & de Lima, G. J. M. M. (2018). Efficacy of energy supplementation on growth performance and immune response of suckling pigs. *Journal of Animal Science*, 96(11), 4723-4730. doi:10.1093/jas/sky335

Marchandin, H., Teyssier, C., Campos, J., Jean-Pierre, H., Roger, F., Gay, B., . . . Jumas-Bilak, E. (2010). *Negativicoccus succinicivorans* gen. nov., sp. nov., isolated from human clinical samples, emended description of the family Veillonellaceae and description of *Negativicutes classis* nov., Selenomonadales ord. nov. and Acidaminococcaceae fam. nov. In the bacterial phylum Firmicutes. *International Journal of Systematic and Evolutionary Microbiology*, 60(Pt6), 1271-1279. doi:10.1099/ijs.0.013102-0

Marounek, M., Skřivanová, E., & Skřivanová, V. (2004). A note on the effect of caprylic acid and triacylglycerols of caprylic and capric acid on growth rate and shedding of coccidia oocysts in weaned piglets. *Journal of Animal and Feed Sciences*, 13(2), 269-274. doi:10.22358/jafs/67411/2004

Marten, B., Pfeuffer, M., & Schrezenmeir, J. (2006). Medium-chain triglycerides. *International Dairy Journal*, 16(11), 1374-1382. doi:10.1016/j.idairyj.2006.06.015

Martínez-Vallespín, B., Vahjen, W., & Zentek, J. (2016). Effects of medium-chain fatty acids on the structure and immune response of IPEC-J2 cells. *Cytotechnology*, 68(5), 1925-1936. doi:10.1007/s10616-016-0003-1

Martín-Peláez, S., Costabile, A., Hoyles, L., Rastall, R. A., Gibson, G. R., La Ragione, R. M., . . . Martín-Orúe, S. M. (2010). Evaluation of the inclusion of a mixture of organic acids or lactulose into the feed of pigs experimentally challenged with *Salmonella* Typhimurium. *Veterinary Microbiology*, 142(3-4), 337-345. doi:10.1016/j.vetmic.2009.09.061

Mateu, E., & Martin, M. (2001). Why is anti-microbial resistance a veterinary problem as well? *Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health*, 48(8), 569-581. doi:10.1111/j.1439-0450.2001.00475.x

Mazzoni, M., Le Gall, M., De Filippi, S., Minieri, L., Trevisi, P., Wolinski, J., . . . Bosi, P. (2008). Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. *The Journal of Nutrition*, 138(8), 1426-1431. doi:10.1093/jn/138.8.1426

McGlone, J. J. (2013). The future of pork production in the world: Towards sustainable, welfare-positive systems. *Animals*, 3, 401-415. doi:10.3390/ani3020401

McLamb, B. L., Gibson, A. J., Overman, E. L., Stahl, C., & Moeser, A. J. (2013). Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic *E. coli* challenge and exacerbates intestinal injury and clinical disease. *PLoS One*, 8, e59838. doi:10.1371/journal.pone.0059838

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, 8(4), e61217. doi:10.1371/journal.pone.0061217

McMurdie, P. J., & Holmes, S. (2014). Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Computational Biology*, 10(4), e1003531. doi:10.1371/journal.pcbi.1003531

Melo, A. D., Silveira, H., Bortoluzzi, C., Lara, L. J., Garbossa, C. A., Preis, G., . . . Rostagno, M. H. (2016). Intestinal alkaline phosphatase and sodium butyrate may be beneficial in attenuating LPS-induced intestinal inflammation. *Genetic and Molecular Research*, 15(4). doi:10.4238/gmr15048875

Messens, W., Goris, J., Dierick, N., Herman, L., & Heyndrickx, M. (2010). Inhibition of *Salmonella* Typhimurium by medium-chain fatty acids in an *in vitro* simulation of the porcine cecum. *Veterinary Microbiology*, 141(1-2), 73-80. doi:10.1016/j.vetmic.2009.08.002

Metzler-Zebeli, B. U., Zijlstra, R. T., Mosenthin, R., & Gänzle, M. G. (2011). Dietary calcium phosphate content and oat β-glucan influence gastrointestinal microbiota, butyrate-producing bacteria and butyrate fermentation in weaned pigs. *FEMS Microbiology Ecology*, 75(3), 402-413. doi:10.1111/j.1574-6941.2010.01017.x

Metzler-Zebeli, B. U., Mann, E., Schmitz-Esser, S., Wagner, M., & Zebeli, Q. (2013). Changing dietary calcium-phosphorus level and cereal source selectively alters abundance of bacteria and metabolites in the upper gastrointestinal tracts of weaned pigs. *Applied and Environmental Microbiology*, 79(23), 7264-7272. doi:10.1128/AEM.02691-13

Michiels, J., Missotten, J., Rasschaert, G., Dierick, N., Heyndrickx, M., & De Smet, S. (2012). Effect of organic acids on *Salmonella* colonization and shedding in weaned piglets in a seeder model. *Journal of Food Protection*, 75(11), 1974-1983. doi:10.4315/0362-028X.JFP-12-210

Michiels, J., De Vos, M., Missotten, J., Ovyn, A., De Smet, S., & Van Ginneken, C. (2013). Maturation of digestive function is retarded and plasma antioxidant capacity lowered in fully weaned low birth weight piglets. *British Journal of Nutrition*, 109(1), 65-75. doi:10.1017/S0007114512000670

Miles, J. M., Cattalini, F. W., Sharbrough, L. E., Wold, L. E., Wharen, J. E., Gerich, J. E., & Haymond, M. W. (1991). Metabolic and neurologic effects of an intravenous medium-chain triglyceride

emulsion. *Journal of Parenteral and Enteral Nutrition*, 15(1), 37-41. doi:10.1177/014860719101500137

Miller, D. W., Prosser, Z., Chee, E. Y., Hansen, C. F., Dunshea, F. R., Mullan, B. P., & Pluske, J. R. (2016). Dietary stimulation of the endogenous somatotropic axis in weaner and grower-finisher pigs using medium chain triglycerides and cysteamine hydrochloride. *Journal of Animal Science and Biotechnology*, 7, 61. doi:10.1186/s40104-016-0121-9

Milo, L. A., Reardon, K. A., & Tappenden, K. A. (2002). Effects of short-chain fatty acid-supplemented total parenteral nutrition on intestinal pro-inflammatory cytokine abundance. *Digestive Diseases and Sciences*, 47(9), 2049-2055. doi:10.1023/A:1019676929875

Min, Y. W., & Rhee, P. L. (2015). The role of microbiota on the gut immunology. *Clinical Therapeutics*, 37(5), 968-975. doi:10.1016/j.clinthera.2015.03.009

Moeser, A. J., Pohl, C. S., & Rajput, M. (2017). Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. *Animal Nutrition*, 3(4), 313-321. doi:10.1016/j.aninu.2017.06.003

Molatová, Z., Skřivanová, E., Baré, J., Houf, K., Bruggeman, G., & Marounek, M. (2011). Effect of coated and non-coated fatty acid supplementation on broiler chickens experimentally infected with *Campylobacter jejuni*. *Journal of Animal Physiology and Animal Nutrition*, 95(6), 701-706. doi:10.1111/j.1439-0396.2010.01100.x

Molist, F., Manzanilla, E. G., Pérez, J. F., & Nyachoti, C. M. (2012). Coarse, but not finely ground, dietary fibre increases intestinal Firmicutes:Bacteroidetes ratio and reduces diarrhoea induced by experimental infection in piglets. *British Journal of Nutrition*, 108(1), 9-15. doi:10.1017/S0007114511005216

Mon, K. K., Saelao, P., Halstead, M. M., Chanthavixay, G., Chang, H. C., Garas, L., . . . Zhou, H. (2015). *Salmonella enterica* serovars Enteritidis infection alters the indigenous microbiota diversity in young layer chick. *Frontiers in Veterinary Medicine*, 2, 61. doi:10.3389/fvets.2015.00061

Montagne, L., Boudry, G., Favier, C., Le Huërou-Luron, I., Lallès, J. P., & Sève, B. (2007). Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *British Journal of Nutrition*, 97(1), 45-57. doi:10.1017/S000711450720580X

Moquet, P. C. A., Onrust, L., Van Immerseel, F., Ducatelle, R., Hendriks, W. H., & Kwakkel, R. P. (2016). Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. *World's Poultry Science Journal*, 72(1), 61-80. doi:10.1017/S004393391500269X

Moredo, F. A., Piñeyro, P., Márquez, G. C., Sanz, M., Colello, R., Etcheverría, A., . . . Leotta, G. A. (2015). Enterotoxigenic *Escherichia coli* subclinical infection in pigs: Bacteriological and genotypic characterization and antimicrobial resistance profiles. *Foodborne Pathogens and Disease*, 12(8), 704-711. doi:10.1089/fpd.2015.1959

Motta, V., Trevisi, P., Bertolini, F., Ribani, A., Schiavo, G., Fontanesi, L., & Bosi, P. (2017). Exploring gastric abcterial community in young pigs. *PLoS One*, 12(3), e0173029. doi:10.1371/journal.pone.0173029

Mroz, Z., Reese, D. E., Øverland, M., van Diepen, J. T., & Kogut, J. (2002). The effects of potassium diformate and its molecular constituents on the apparent ileal and fecal digestibility and retention of nutrients in growing-finishing pigs. *Journal of Anim*al *Science*, 80(3), 681-690. doi:10.2527/2002.803681x

Mroz, Z. (2005). Organic acids as potential alternatives to antibiotic growth promoters for pigs. *Advances in Pork Production*, 16, 169-182.

Mroz, Z., Koopmans, S. J., Bannink, A., Partanen, K., Krasucki, W., Øverland, M., & Radcliffe, S. (2006). Chapter 4 Carboxylic acids as bioregulators and gut growth promoters in nonruminants. In R. Mosenthin, J. Zentek, & T. Zebrowska (Eds.), *Biology of Growing Animals* (ISBN 978-0-444-51232-1, pp. 81-133). Elsevier Ltd. doi:10.1016/S1877-1823(09)70091-8

Mu, C., Yang, Y., Su, Y., Zoetendal, E. G., & Zhu, W. (2017). Differences in microbiota membership along the gastrointestinal tract of piglets and their differential alterations following an early-life antibiotic intervention. *Frontiers in Microbiology*, 8, 797. doi:10.3389/fmicb.2017.00797

Nair, K. S., Welle, S. L., Halliday, D., & Campbell, R. G. (1988). Effect of beta-hydroxybutyrate on whole-body leucine kinetics and fractional mixed skeletal muscle protein synthesis in humans. *The Journal of Clinical Investigation*, 82(1), 198-205. Doi:10.1172/JCI113570

Nakatani, M., Inoue, R., Tomonaga, S., Fukuta, K., & Tsukahara, T. (2018). Production, absorption, and blood flow dynamics of short-chain fatty acids produced by fermentation in piglet hindgut during the suckling–weaning period. *Nutrients*, 10(9), 1220. doi:10.3390/nu10091220

Namkung, H., Yu, H., Gong, J., & Leeson, S. (2011). Antimicrobial activity of butyrate glycerides toward *Salmonella* Typhimurium and *Clostridium perfringens*. *Poultry Science*, 90(10), 2217-2222. doi:10.3382/ps.2011-01498

NASA. (2012, October). Technology readiness level. Retrieved from https://www.nasa.gov/directorates/heo/scan/engineering/technology/txt_accordion1.html

Naughton, J. A., Mariño, K., Dolan, B., Reid, C., Gough, R., Gallagher, M. E., . . . Clyne, M. (2013). Divergent mechanisms of interaction of *Helicobacter pylori* and *Campylobacter jejuni* with mucus and mucins. *Infection and Immunity*, 81(8), 2838-2850. doi:10.1128/IAI.00415-13

Nazer, A. I., Kobilinsky, A., Tholozan, J. L., & Dubois-Brissonnet, F. (2005). Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: A synergistic effect? *Food Microbiology*, 22(5), 391-398. doi:10.1016/j.fm.2004.10.003

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., & Bugarski, B. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science*, 1, 1806-1815. doi:10.1016/j.profoo.2011.09.265

Newman, M. A., Petri, R. M., Grüll, D., Zebeli, Q., & Metzler-Zebeli, B. U. (2018). Transglycosylated starch modulates the gut microbiome and expression of genes related to lipid synthesis in liver and adipose tissue of pigs. *Frontiers in Microbiology*, 9, 224. doi:10.3389/fmicb.2018.00224

Nishi, Y., Hiejima, H., Hosoda, H., Kaiya, H., Mori, K., Fukue, Y., . . . Kojima, M. (2005). Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. *Endocrinology*, 146(5), 2255-2264. doi:10.1210/en.2004-0695

Niu, Q., Li, P., Hao, S., Zhang, Y., Kim, S. W., Li, H., . . . Huang, R. (2015). Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. *Scientific Reports*, 5, 9938. doi:10.1038/srep09938

Nofrarías, M., Manzanilla, E. G., Pujols, J., Gibert, X., Majó, N., Segalés, J., & Gasa, J. (2006). Effects of spray-dried porcine plasma and plant extracts on intestinal morphology and on leukocyte cell subsets of weaned pigs. *Journal of Animal Science*, 84(10), 2735-2742. doi:10.2527/jas.2005-414

NRC. (2012). Nutrient requirements of swine: Eleventh revised edition (11th ed., ISBN 978-0-309-22423-9). Washington, DC: The National Academies Press. doi:10.17226/13298

nutriNews. (2018). Tabla ácidos orgánicos y/o grasos 2018. Retrieved from file:///C:/Users/Paola/Downloads/Tabla_AAOO-Grasos_nutriNews2018.pdf

Ocasio-Vega, C., Delgado, R., Abad-Guamán, R., Menoyo, D., Nicodemus, N., Carabaño, R., . . . García, J. (2016). Efecto de la suplementación con *Bacillus amyloliquefaciens* y heptanoato sódico sobre los parámetros de crecimiento en conejos de cebo. *XLI Symposium de Cunicultura*, 78-79.

Odle, J., Benevenga, N. J., & Crenshaw, T. D. (1992). Evaluation of [1-^{14}C]-medium-chain fatty acid oxidation by neonatal piglets using continuous-infusion radiotracer kinetic methodology. *The Journal of Nutrition*, 122(11), 2183-2189. doi:10.1093/jn/122.11.2183

O'Hara, A. M., & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Reports*, 7(7), 688-693. doi:10.1038/sj.embor.7400731

OiE. (2016, November). The OiE strategy on antimicrobial resistance and prudent use of antimicrobials. Retrieved from http://www.oie.int/fileadmin/Home/eng/Media_Center/docs/pdf/PortailAMR/EN_OIE-AMRstrategy.pdf

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., . . . Wagner, H. (2017). vegan: Community ecology Package. Retrieved from https://CRAN.R-project.org/package=vegan

Omonijo, F. A., Kim, S., Guo, T., Wang, Q., Gong, J., Lahaye, L., . . . Yang, C. (2018). Development of novel microparticles for effective delivery of thymol and lauric acid to pig intestinal tract. *Journal of Agricultural and Food Chemistry*, 66(37), 9608-9615. doi:10.1021/acs.jafc.8b02808

Onrust, L., Van Driessche, K., Ducatelle, R., Schwarzer, K., Haesebrouck, F., & Van Immerseel, F. (2018). Valeric acid glyceride esters in feed promote broiler performance and reduce the incidence of necrotic enteritis. *Poultry Science*, 97(7), 2303-2311. doi:10.3382/ps/pey085

Ooyama, K., Kojima, K., Aoyama, T., & Takeuchi, H. (2009). Decrease of food intake in rats after ingestion of medium-chain triacylglycerol. *Journal of Nutritional Science and Vitaminology*, 55(5), 423-427.

Orhan, G., Bayram, A., Zer, Y., & Balci, I. (2005). Synergy tests by E test and checkerboard methods of antimicrobial combinations against *Brucella melitensis*. *Journal of Clinical Microbiology*, 43(1), 140-143. doi:10.1128/JCM.43.1.140-143.2005

Øverland, M., Granli, T., Kjos, N. P., Fjetland, O., Steien, S. H., & Stokstad, M. (2000). Effect of dietary formates on growth performance, carcass traits, sensory quality, intestinal microflora, and stomach alterations in growing-finishing pigs. *Journal of Animal Science*, 78(7), 1875-1884.

Øverland, M., Kjos, N. P., Borg, M., & Sørum, H. (2007). Organic acids in diets for entire male pigs. *Livestock Science*, 109(1-3), 170-173. doi:10.1016/j.livsci.2007.02.002

Øverland, M., Kjos, N. P., Borg, M., Skjerve, E., & Sørum, H. (2008). Organic acids in diets for entire male pigs: Effect on skatole level, microbiota in digesta, and growth performance. *Livestock Science*, 115(2-3), 169-178. doi:10.1016/j.livsci.2007.07.007

Pajarillo, E. A. B., Chae, J. P., Balolong, M. P., Kim, H. B., & Kang, D. K. (2014). Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. The *Journal of General and Applied Microbiology*, 60(4), 140-146. doi:10.2323/jgam.60.140

Parra Huertas, R. A. (2010). Revisión: Microencapsulación de alimentos. *Revista Facultad Nacional de Agronomía - Medellín*, 63(2), 5669-5684. doi:10.15446/rfnam

Partanen, K. H., & Mroz, Z. (1999). Organic acids for performance enhancement in pig diets. *Nutrition Research Reviews*, 12(1), 117-145. doi:10.1079/095442299108728884

Partanen, K. (2001). Organic acids – their efficacy and modes of action in pigs. In A. Piva, K. E. Knudsen, & J. E. Lindberg (Eds.), *Gut environment in pigs* (pp. 201-217). Nottingham, UK: The Nottingham University Press.

Partanen, K., Siljander-Rasi, H., Alaviuhkola, T., Suomi, K., & Fossi, M. (2002a). Performance of growing–finishing pigs fed medium- or high-fibre diets supplemented with avilamycin, formic acid or formic acid–sorbate blend. *Livestock Production Science*, 73(2-3), 139-152. doi:10.1016/S0301-6226(01)00255-X

Partanen, K., Siljander-Rasi, H., & Suomi, K. (2002b). Dietary preferences of weaned piglets offered diets containing organic acids. *Agricultural and Food Science*, 11(2), 107-119. doi:10.23986/afsci.5718

Partanen, K., & Jalava, T. (2005). Effects of some organic acids and salts on microbial fermentation in the digestive tract of piglets estimated using an *in vitro* gas production technique. *Agricultural and Food Science*, 14(9), 311-324. doi:10.2137/145960605775897687

Partanen, K., Siljander-Rasi, H., Pentikäinen, J., Pelkonen, S., & Fossi, M. (2007). Effects of weaning age and formic acid-based feed additives on pigs from weaning to slaughter. *Archives of Animal Nutrition*, 61(5), 336-356. doi:10.1080/17450390701556866

Peffer, P. L., Lin, X., & Odle, J. (2005). Hepatic β-oxidation and carnitine palmitoyltransferase I in neonatal pigs after dietary treatments of clofibric acid, isoproterenol, and medium-chain triglycerides. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, 288(6), R1518-R1524. doi:10.1152/ajpregu.00822.2004

Peng, L., Li, Z. R., Green, R. S., Holzman, I. R., & Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in caco-2 cell monolayers. *The Journal of Nutrition*, 139(9), 1619-1625. doi:10.3945/jn.109.104638

Perdigón, G., Fuller, R., & Raya, R. (2001). Lactic acid bacteria and their effect on the immune system. *Current Issues in Intestinal Microbiology*, 2(1), 27-42.

Petri, D., Hill, J. E., & Van Kessel, A. G. (2010). Microbial succession in the gastrointestinal (GIT) of the preweaned pig. *Livestock Science*, 133(1-3), 107-109. doi:10.1016/j.livsci.2010.06.037

Petschow, B. W., Batema, R. P., & Ford, L. L. (1996). Susceptibility of *Helicobacter pylori* to bactericidal properties of medium-chain monoglycerides and free fatty acids. *Antimicrobial Agents and Chemotherapy*, 40(2), 302-306.

Pié, S., Lallès, J. P., Blazy, F., Laffitte, J., Sève, B., & Oswald, I. P. (2004). Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *The Journal of Nutrition*, 134(3), 641-647. doi:10.1093/jn/134.3.641

Pieper, R, Villodre Tudela, C., Taciak, M., Bindelle, J., Pérez, J. F., & Zentek, J. (2016). Health relevance of intestinal protein fermentation in young pigs. *Animal Health Research Reviews*, 17(2), 137-147. doi:10.1017/S1466252316000141

Piñeiro, C., Piñeiro, M., Morales, J., Andrés, M., Lorenzo, E., Pozo, M. D., . . . Lampreave, F. (2009). Pig-MAP and haptoglobin concentration reference values in swine from commercial farms. *The Veterinary journal*, 179(1), 78-84. doi:10.1016/j.tvjl.2007.08.010

Piva, A., Morlacchini, M., Casadei, G., Gatta, P. P., Biagi, G., & Prandini, A. (2002). Sodium butyrate improves growth performance of weaned piglets during the first period after weaning. *Italian Journal of Animal Science*, 1(1), 35-41. doi:10.4081/ijas.2002.35

Piva, A., Pizzamiglio, V., Morlacchini, M., Tedeschi, M., & Piva, G. (2007). Lipid microencapsulation allows slow release of organic acids and natural identical flavors along the swine intestine. *Journal of Animal Science*, 85(2), 486-493. doi:10.2527/jas.2006-323

Pluske, J. R., Kerton, D. J., Cranwell, P. D., Campbell, R. G., Mullan, B. P., King, R. H., . . . Dunshea, F. R. (2003). Age, sex, and weight at weaning influence organ weight and gastrointestinal development of weanling pigs. *Australian Journal of Agricultural Research*, 54(5), 515-527. doi:10.1071/AR02156

Poeikhampha, T., & Bunchasak, C. (2011). Comparative effects of sodium gluconate, mannan oligosaccharide and potassium diformate on growth performances and small intestinal norphology of nursery pigs. *Asian-Australasian Journal of Agricultural Research*, 24(6), 844-850. doi:10.5713/ajas.2011.10334

Pollock, J., Gally, D. L., Glendinning, L., Tiwari, R., Hutchings, M. R., & Houdijk, J. G. M. (2018). Analysis of temporal fecal microbiota dynamics in weaner pigs with and without exposure to enterotoxigenic *Escherichia coli*. *Journal of Animal Science*, 96, 3777-3790. doi:10.1093/jas/sky260

Polycarpo, G. V., Andretta, I., Kipper, M., Cruz-Polycarpo, V. C., Dadalt, J. C., Rodrigues, P. H. M., & Albuquerque, R. (2017). Meta-analytic study of organic acids as an alternative performance-

enhancing feed additive to antibiotics for broiler chickens. *Poultry Science*, 96(10), 3645-3653. doi:10.3382/ps/pex178

Poole, T. L., Suchodolski, J. S., Callaway, T. R., Farrow, R. L., Loneragan, G. H., & Nisbet, D. J. (2013). The effect of chlortetracycline on faecal microbial populations in growing swine. *Journal of Global Antimicrobial Resistance*, 1(3), 171-174. doi:10.1016/j.jgar.2013.04.004

Poulsen, A. R., Jonge, N., Nielsen, J. L., Højberg, O., Lauridsen, C., Cutting, S. M., & Canibe, N. (2018). Impact of *Bacillus* spp. spores and gentamicin on the gastrointestinal microbiota of suckling and newly weaned piglets. *PLos One*, 13(11), e0207382. doi:10.1371/journal.pone.0207382

Pozuelo, M., Panda, S., Santiago, A., Mendez, S., Accarino, A., Santos, J., . . . Manichanh, C. (2015). Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome. *Scientific Reports*, 5, 12693. doi:10.1038/srep12693

Prigitano, A., Romanò, L., Auxilia, F., Castaldi, S., & Tortorano, A. M. (2018). Antibiotic resistance: Italian awareness survey 2016. *Journal of Infection and Public Health*, 11(1), 30-34. doi:10.1016/j.jiph.2017.02.010

Prohászka, L. (1980). Antibacterial effect of volatile fatty acids in enteric *E. coli*-infections of rabbits. *Zentralblatt für Veterinärmedizin. Reihe B*, 27(8), 631-639. doi:10.1111/j.1439-0450.1980.tb01726.x

PSU. (2009). The virtual nature trail at Penn State New Kensington. Ecological succession. Retrieved from https://www.psu.edu/dept/nkbiology/naturetrail/succession.htm

PubChem. (2019, January). National Institutes of Health (NIH). PubChem. Retrieved from https://pubchem.ncbi.nlm.nih.gov/

Putnam, D. E., & Garrett, J. E. (2005). Microencapsulation: Opportunities to improve animal products and nutrition. Retrieved from http://www.dairyweb.ca/Resources/PNWANC2005/Putnam.pdf

R Core Team. (2013). R: A language and environment for statistical computing (ISBN 3-900051-07-0). Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Ramayo-Caldas, Y., Mach, N., Lepage, P., Levenez, F., Denis, C., Lemonnier, G., . . . Estellé, J. (2016). Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. *The ISME Journal*, 10(12), 2973-2977. doi:10.1038/ismej.2016.77

Ranjan, R., Rani, A., Metwally, A., McGee, H. S., & Perkins, D. L. (2016). Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon. *Biochemical and Biophysical Research Communications*, 469(4), 967-977. doi:10.1016/j.bbrc.2015.12.083

Rasschaert, G., Michiels, J., Tagliabue, M., Missotten, J., De Smet, S., & Heyndrickx, M. (2016). Effect of organic acids on *Salmonella* shedding and colonization in pigs on a farm with high *Salmonella* prevalence. *Journal of Food Protection*, 79(1), 51-58. doi:10.4315/0362-028X.JFP-15-183

Rhouma, M., Fairbrother, J. M., Thériault, W., Beaudry, F., Bergeron, N., Laurent-Lewandowski, S., & Letellier, A. (2017). The fecal presence of enterotoxin and F4 genes as an indicator of efficacy of treatment with colistin sulfate in pigs. *BMC Microbiology*, 17(1), 6. doi:10.1186/s12866-016-0915-0

Richardson, A. J., Calder, A. G., Stewart, C. S., & Smith, A. (1989). Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas chromatography. *Letters in Applied Microbiology*, 9(1), 5-8. doi:10.1111/j.1472-765X.1989.tb00278.x

Rideout, J. R., He, Y., Navas-Molinas, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., . . . Caporaso, J. G. (2014). Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ*, 2, e545. doi:10.7717/peerj.545

Rintala, A., Pietilä, S., Munukka, E., Eerola, E., Pursiheimo, J. P., Laiho, A., . . . Huovinen, P. (2017). Gut microbiota analysis results are highly dependent on the 16S rRNA gene target region, whereas the impact of DNA extraction is minor. *Journal of Biomolecular Techniques*, 28(1), 19-30. doi:10.7171/jbt.17-2801-003

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C. G., & Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Frontiers in Microbiology*, 7, 185. doi:10.3389/fmicb.2016.00185

Risley, C. R., Kornegay, E. T., Lindemann, M. D., Wood, C. M., & Eigel, W. N. (1993). Effect of feeding organic acids on gastrointestinal digesta measurements af various times postweaning in pigs challenged with enterotoxigenic *Escherichia coli*. *Canadian Journal of Animal Science*, 73(4), 931-940. doi:10.4141/cjas93-094

Roca, M., Nofrarías, M., Majó, N., María, A., Pérez de Rozas, A. M., Segalés, J., . . . Badiola, I. (2014). Changes in bacterial population of gastrointestinal tract of weaned pigs fed with different additives. *BioMed Research International*, 269402. doi:10.1155/2014/269402

Rodas, B. D., & Maxwell, C. V. (1990). The effect of fat source and medium-chain triglyceride level on performance of the early-weaned pig. *Animal Science Research Report*, MP-129(ref.9), 278-287.

Rostami, Z., Ahmad, M. A., Khan, M. U., Mishra, A. P., Rashidzadeh, S., & Shariati, M. A. (2016). Food preservation by hurdle technology: A review of different hurdle and interaction with focus on foodstuffs. *Journal of Pure and Applied Microbiology*, 10(4), 2633-2639. doi:10.22207/JPAM.10.4.20

Roth, F. X., & Kirchgessner, M. (1998). Organic acids as feed additives for young pigs: Nutritional and gastrointestinal effects. *Journal of Animal and Feed Sciences*, 7(S1), 25-33. doi:10.22358/jafs/69953/1998

Ruiz, V. L. A., Bersano, J. G., Carvalho, A. F., Catroxo, M. H. B., Chiebao, D. P., Gregori, F., . . . Tonietti, P. O. (2016). Case-control study of pathogens involved in piglet diarrhea. *BMC Research Notes*, 11(9), 22. doi:10.1186/s13104-015-1751-2

Ryan, M. P., O'Dwyer, J., & Adley, C. C. (2017). Evaluation of the complex nomenclature of the clinically and veterinary significant pathogen *Salmonella*. *BioMed Research International*, 3782182. doi:10.1155/2017/3782182

Saco, Y., Fraile, L., Giménez, M., Alegre, A., López-Jimenez, R., Cortey, M., . . . Bassols, A. (2011). Serum acute phase proteins as biomarkers of pleuritis and cranio-ventral pulmonary consolidation in slaughter-aged pigs. *Research in Veterinary Science*, 91(1), 52-57. doi:10.1016/j.rvsc.2010.08.016

Salazar, L. C., Ko, H. L., Yang, C. H., Llonch, L., Manteca, X., Camerlink, I., & Llonch, P. (2018). Early socialisation as a strategy to increase piglets' social skills in intensive farming conditions. *Applied Animal Behaviour Science*, 206, 25-31. doi:10.1016/j.applanim.2018.05.033

Sanschagrin, S., & Yergeau, E. (2014). Next-generation sequencing of 16S ribosomal RNA gene amplicons. *Journal of Visualized Experiments*, 90, e51709. doi:10.3791/51709

Sansonetti, P. (2002). Host-pathogen interactions: the seduction of molecular cross talk. *Gut*, 50(S3), iii2-iii8. doi:10.1136/gut.50.suppl_3.iii2

Schmitt, M. G., Soergel, K. H., & Wood, C. M. (1976). Absorption of short chain fatty acids from the human jejúnum. *Gastroenterology*, 70(2), 211-215.

Schneider, D., Thürmer, A., Gollnow, K., Lugert, R., Gunka, K., Gross, U., & Daniel, R. (2017). Gut bacterial communities of diarrheic patients with indications of *Clostridioides difficile* infection. *Scientific Data*, 4, 170152.

Schönfeld, P., & Wojtczak, L. (2016). Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. *Journal of Lipid Research*, 57(6), 943-954. doi:10.1194/jlr.R067629

Schroyen, M., Stinckens, A., Verhelst, R., Niewold, T., & Buys, N. (2012). The search for the gene mutations underlying enterotoxigenic *Escherichia coli* F4ab/ac susceptibility in pigs: a review. *Veterinary Research*, 43, 70. doi:10.1186/1297-9716-43-70

Shannon, C. E. (1963). The mathematical theory of communication. *The Bell System Technical Journal*, 27, 379-423, 623-656.

Shannon, C. E., & Weaver, W. (1963). The mathematical theory of communication. (pp. 117). Urbana, IL: University Illinois Press.

Sheu, C. W., & Freese, E. (1972). Effect of fatty acids on growth and envelope proteins of *Bacillus subtilis*. *Journal of Bacteriology*, 111(2), 516-524.

Shilling, M., Matt, L., Rubin, E., Visitacion, M. P., Haller, N. A., Grey, S. F., & Woolverton, C. J. (2013). Antimicrobial effects of virgin coconut oil and its medium-chain fatty acids on *Clostridium difficile*. *Journal of Medicinal Food*, 16(12), 1079-1085. doi:10.1089/jmf.2012.0303

Sims, D., Sudbery, I., Ilott, N. E., Heger, A., & Ponting, C. P. (2014). Sequencing depth and coverage: Key considerations in genomic analyses. *Nature Reviews*. Genetics, 15(2), 121-132. doi:10.1038/nrg3642

Singh, M. N., Hemant, K. S. Y., Ram, M., & Shivakumar, H. G. (2010). Microencapsulation: A promising technique for controlled drug delivery. *Research in Pharmaceutical Sciences*, 5(2), 65-77.

Skrivanová, E., Savka, O. G., & Marounek, M. (2004). *In vitro* effect of C2-C18 fatty acids on Salmonellas. *Folia Microbiologica*, 49(2), 199-202.

Skrivanová, E., Marounek, M., Benda, V., & Brezina, P. (2006). Susceptibility of *Escherichia coli*, *Salmonella* sp. and *Clostridium perfringens* to organic acids and monolaurin. *Veterinarni Medicina*, 51(3), 81-88.

Slifierz, M. J., Friendship, R., de Lange, C. F., Rudar, M., & Farzan, A. (2013). An epidemiological investigation into the association between biomarkers and growth performance in nursery pigs. *BMC Veterinary Research*, 9, 247. doi:10.1186/1746-6148-9-247

Smith, B., Li, N., Andersen, A. S., Slotved, H. C., & Krogfelt, K. A. (2011). Optimising bacterial DNA extraction from faecal samples: Comparison of three methods. *The Open Microbiology Journal*, 5, 14-17. doi:10.2174/1874285801105010014

Smith, D. J., Barri, A., Herges, G., Hahn, J., Yersin, A. G., & Jourdan, A. (2012). *In vitro* dissolution and *in vivo* abspoption of calcium [1-^{14}C]butyrate in free or protected forms. *Journal of Agricultural and Food Chemistry*, 60(12), 3151-3157. doi:10.1021/jf3001058

Solano-Aguilar, G. I., Lakshman, S., Jang, S., Beshah, E., Xie, Y., Sikaroodi, M., . . . Davis, C. D. (2018). The effect of feeding cocoa powder and *Lactobacillus rhamnosus* LGG on the composition and function of pig intestinal microbiome. *Current Developments in Nutrition*, 2(5), nzy011. doi:10.1093/cdn/nzy011

Soler, C., Goossens, T., Bermejo, A., Migura-García, L., Cusco, A., Francino, O., & Fraile, L. (2018). Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. *PLoS One*, 13(5), e0197353. doi:10.1371/journal.pone.0197353

Sonarome. (2014, December). Spray drying technology. Retrieved from http://sonarome.com/2014/12/02/spray-drying-technology/

Spitzer, F., Vahjen, W., Pieper, R., Martínez-Vallespín, B., & Zentek, J. (2014). A standardised challenge model with an enterotoxigenic F4+ *Escherichia coli* strain in piglets assessing clinical traits and faecal shedding of fae and est-II toxin genes. *Archives of Animal Nutrition*, 68(6), 448-459. doi:10.1080/1745039X.2014.968701

Stecher, B., Chaffron, S., Käppeli, R., Hapfelmeier, S., Freedrich, S., Weber, T. C., . . . Hard, W. D. (2010). Like will to like: Abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. *PLoS Pathogens*, 6(1), e1000711. doi:10.1371/journal.ppat.1000711

Stein, J. L., Marsh, T. L., Wu, K. Y., Shizuya, H., & DeLong, E. F. (1996). Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. *Journal of Bacteriology*, 178(3), 591-599. doi:10.1128/jb.178.3.591-599.1996

Stensland, I., Kim, J. C., Bowring, B., Collins, A. M., Mansfield, J. P., & Pluske, J. R. (2015). A comparison of diets supplemented with a feed additive containing organic acids, cinnamaldehyde and a permeabilizing complex, or zinc oxide, on post-weaning diarrhoea, selected bacterial populations, blood measures and performance in weaned pigs experime. *Animals*, 5(4), 1147-1168. doi:10.3390/ani5040403

Straarup, E. M., Danielsen, V., Høy, C. E., & Jakobsen, K. (2006). Dietary structured lipids for post-weaning piglets: Fat digestibility, nitrogen retention and fatty acid profiles of tissues. *Journal of Animal Physiology and Animal Nutrition*, 90(3-4), 124-135. doi:10.1111/j.1439-0396.2005.00573.x

Sturhan, D. (1985). Species, subspecies, race and pathotype problems in nematodes. *EPPO Bulletin*, 15,(2) 139-144. doi:10.1111/j.1365-2338.1985.tb00213.x

Su, X. L., Tian, Q., Zhang, J., Yuan, X. Z., Shi, X. S., Guo, R. B., & Qiu, Y. L. (2014). *Acetobacteroides hydrogenigenes* gen. nov., sp. nov., an anaerobic hydrogen-producing bacterium in the family Rikenellaceae isolated from a reed swamp. *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt9), 2986-2991. doi:10.1099/ijs.0.063917-0

Suau, A., Bonnet, R., Sutren, M., Godon, J. J., Gibson, G. R., Collins, M. D., & Doré, J. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and Environmental Microbiology*, 65(11), 4799-807. doi:10.1186/2049-2618-2-8

Suen, G., Weimer, P. J., Stevenson, D. M., Aylward, F. O., Boyum, J., Deneke, J., . . . Brumm, P. J. (2011). The complete genome sequence of *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic specialist. *PLoS One*, 6(4), e18814. doi:10.1371/journal.pone.0018814

Suiryanrayna, M. V. A. N., & Ramana, J. V. (2015). A review of the effects of dietary organic acids fed to swine. *Journal of Animal Science and Biotechnology*, 6, 45. doi:10.1186/s40104-015-0042-z

Sun, Y., & O'Riordan, M. X. (2013). Regulation of bacterial pathogenesis by intestinal short-chain fatty acids. *Advances in Applied Microbiology*, 85, 93-118. doi:10.1016/B978-0-12-407672-3.00003-4

Sunkara, L. T., Jiang, W., & Zhang, G. (2012). Modulation of antimicrobial host defense peptide gene expression by free fatty acids. *PLoS One*, 7(11), e49558. doi:10.1371/journal.pone.0049558

Suzuki, M., Takaishi, S., Nagasaki, M., Onozawa, Y., Iino, I., Maeda, H., . . . Oda, T. (2013). Medium-chain fatty acid-sensing receptor, GPR84, is a proinflammatory receptor. The *Journal of Biological Chemistry*, 288(15), 10684-10691. doi:10.1074/jbc.M112.420042

Symersky, T., Vu, M. K., Frölich, M., Biemond, I., Masclee, A. A. (2002). The effect of equicaloric medium-chain and long-chain triglycerides on pancreas enzyme secretion. *Clinical Physiology and Funcional Imaging*, 22(5), 307-311.

Tangwatcharin, P., & Khopaibool, P. (2012). Activity of virgin coconut oil, lauric acid or monolaurin in combination with lactic acid against *Staphylococcus aureus*. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 43(4), 969-985.

Taube, V. A., Neu, M. E., Hassan, Y., Verspohl, J., Beyerbach, M., & Kamphues, J. (2009). Effects of dietary additives (potassium diformate/organic acids) as well as influences of grinding intensity (coarse/fine) of diets for weaned piglets experimentally infected with *Salmonella* Derby or *Escherichia coli*. *Journal of Animal Physiology and Animal Nutrition*, 93(3), 350-358. doi:10.1111/j.1439-0396.2008.00894.x

Thacker, P. A., Campbelll, G. L., & GrootWassink, J. (1992). The effect of organic acids and enzyme supplementation on the performance of pigs fed barley-based diets. *Canadian Journal of Animal Science*, 72(2), 395-402. doi:10.4141/cjas92-047

Thompson, S., Miller, K., & López, A. (2007). Cocoa and coffee food microbiology. In M. P. Doyle, & L. R. Beuchat (Eds.), *Food Microbiology: Fundamentals and Frontiers* (3th ed., pp. 837-850). Washington, DC: ASM Press. doi:10.1128/9781555818463.ch3

Trevisi, P., Priori, D., Motta, V., Luise, D., Jansman, A. J. M., Koopmans, S. J., & Bosi, P. (2017). The effects of starter microbiota and the early life feeding of medium chain triglycerides on the gastric transcriptome profile of 2- or 3-week-old cesarean delivered piglets. *Journal of Animal Science and Biotechnology*, 8, 82. doi:10.1186/s40104-017-0213-1

Tsiloyiannis, V. K., Kyriakis, S. C., Vlemmas, J., & Sarris, K. (2001). The effect of organic acids on the control of porcine post-weaning diarrhoea. *Research in Veterinary Science*, 70(3), 287-293. doi:10.1053/rvsc.2001.0476

Turner, J. L., Dritz, S. S., & Minton, J. E. (2001). REVIEW: Alternatives to conventional antimicrobials in swine diets. *The Professional Animal Scientist*, 17(4), 217-226. doi:10.15232/S1080-7446(15)31633-8

Turner, J. M., Josephson, J., Field, C. J., Wizzard, P. R., Ball, R. O., Pencharz, P. B., & Wales, P. W. (2016). Liver disease, systemic inflammation, and growth using a mixed parenteral lipid emulsion, containing soybean oil, fish oil, and medium chain triglycerides, compared with soybean oil in parenteral nutrition-fed neonatal piglets. *Journal of Parenteral and Enteral Nutrition*, 40(7), 973-981. doi:10.1177/0148607115579711

Umpleby, A. M., Chubb, D., Boroujerdi, M. A., & Sonksen, P. H. (1988). The effect of ketone bodies on leucine and alanine metabolism in dogs. *Clinical Science*, 74(1), 41-48.

Upadhaya, S. D., Lee, K. Y., & Kim, I. H. (2014). Protected organic acid blends as an alternative to antibiotics in finishing pigs. *Asian-Australasian Journal of Animal Sciences*, 27(11), 1600-1607. doi:10.5713/ajas.2014.14356

Upadhaya, S. D., Lee, K. Y., & Kim, I. H. (2016). Effect of protected organic acid blends on growth performance, nutrient digestibility and faecal micro flora in growing pigs. *Journal of Applied Animal Research*, 44(1), 238-242. doi:10.1080/09712119.2015.1031775

Upadhaya, S. D., Lee, K. Y., Serpunja, S., Song, T. H., & Kim, I. H. (2018). Growth performance, nutrient digestibility, fecal microbiota and fecal noxious gas emission in weaning pigs fed high and low density diet with and without protected organic acid blends. *Animal Feed Science and Technology*, 239, 1-8. doi:10.1016/j.anifeedsci.2017.12.013

van Beers-Schreurs, H. M. G., Nabuurs, M. J. A., Vellenga, L., Kalsbeek-van der Valk, H. J., Wensing, J., & Breukink, H. J. (1998). Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood, *The Journal of Nutrition*, 947-953. doi:10.1093/jn/128.6.947

van den Borne, J. J. G. C., Heetkamp, M. J. W., Buyse, J., & Niewold, T. A. (2015). Fat coating of Ca butyrate results in extended butyrate release in the gastrointestinal tract of broilers. *Livestock Science*, 175, 96-100. doi:10.1016/j.livsci.2015.02.016

van der Aar, P. J., Molist, F., & van der Klis, J. D. (2017). The central role of intestinal health on the effect of feed additives on feed intake in swine and poultry. *Animal Feed Science and Technology*, 233, 64-75. doi:10.1016/j.anifeedsci.2016.07.019

van der Poll, T., & van Deventer, S. J. (1999). Cytokines and anticytokines in the pathogenesis of sepsis. *Infectious Disease Clinics of North America*, 13(2), 413-426.

Van Immerseel, F., De Buck, J., Boyen, F., Bohez, L., Pasmans, F., Volf, J., . . . Ducatelle, R. (2004). Medium-chain fatty acids decrease colonization and invasion through *hilA* suppression shortly after infection of chickens with *Salmonella enterica* serovar Enteritidis. *Applied and Environmental Microbiology*, 70(6), 3582-3587. doi:10.1128/AEM.70.6.3582-3587.2004

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74(10), 3583-3597. doi:10.3168/jds.S0022-0302(91)78551-2

Vande Maele, L., Heyndrickx, M., Maes, D., De Pauw, N., Mahu, M., Verlinden, M., . . . Boyen, F. (2016). *In vitro* susceptibility of *Brachyspira hyodysenteriae* to organic acids and essential oil components. *The Journal of Veterinary Medical Science*, 78(2), 325-328. doi:10.1292/jvms.15-0341

Venables, W. N., & Ripley, B. D. (2002). Modern applied statistics with S (4th ed.). New York, NY: Springer. Retrieved from http://www.stats.ox.ac.uk/pub/MASS4

Vigo, G. B., Cappuccio, J. A., Piñeyro, P. E., Salve, A., Machuca, M. A., Quiroga, M. A., . . . Perfumo, C. J. (2009). *Salmonella enterica* subclinical infection: Bacteriological, serological, pulsed-field gel electrophoresis, and antimicrobial resistance profiles—longitudinal study in a three-site farrow-to-finish farm. *Foodborne Pathogens and Disease*, 6(8), 965-972. doi:10.1089/fpd.2008.0239

Villodre Tudela, C., Boudry, C., Stumpff, F., Aschenback, J., Vahjen, W., Zentek, J., & Pieper, R. (2016). Influence of bacterial metabolites on barrier function and pro-inflammatory signalling in epithelial cells *in vitro*. *Proceedings of the Society of Nutrition Physiology*, 25, 23.

Vital, M., Howe, A. C., & Tiedje, J. M. (2014). Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. *MBio*, 5(2), e00889. doi:10.1128/mBio.00889-14

Walia, K., Argüello, H., Lynch, H., Leonard, F. C., Grant, J., Yearsley, D., . . . Lawlor, P. G. (2016). Effect of feeding sodium butyrate in the late finishing period on *Salmonella* carriage, seroprevalence, and growth of finishing pigs. *Preventive Veterinary Medicine*, 131, 79-86. doi:10.1016/j.prevetmed.2016.07.009

Wall, K. M., Diersen-Schade, D., & Innis, S. M. (1994). Plasma and tissue lipids of piglets fed formula containing saturated fatty acids from medium-chain triglycerides with or without fish oil. *The American Journal of Clinical Nutrition*, 59(6), 1317-1324. doi:10.1093/ajcn/59.6.1317

Walsh, M. C., Rostagno, M. H., Gardiner, G. E., Sutton, A. L., Richert, B. T., & Radcliffe, J. S. (2012). Controlling *Salmonella* infection in weanling pigs through water delivery of direct-fed microbials or organic acids. part I: Effects on growth performance, microbial populations, and immune status. *Journal of Animal Science*, 90(1), 261-271. doi:10.2527/jas.2010-3598

Wang, L., Rothemund, D., Curd, H., & Reeves, P. R. (2003). Species-wide variation in the *Escherichia coli* flagellin. *Journal of Bacteriology*, 185(9), 2936-2943. doi:10.1128/JB.185.9.2936

Weber, T. E., & Kerr, B. J. (2008). Effect of sodium butyrate on growth performance and response to lipopolysaccharide in weanling pigs. *Journal of Animal Science*, 86(2), 442-450. doi:10.2527/jas.2007-0499

Weber, T. E., van Sambeek, D. M., Gabler, N. K., Kerr, B. J., Moreland, S., Johal, S., & Edmonds, M. S. (2014). Effects of dietary humic and butyric acid on growth performance and response to lipopolysaccharide in young pigs. *Journal of Animal Science*, 92(9), 4172-4179. doi:10.2527/jas.2013-7402

Weigel, W. A., & Demuth, D. R. (2016). QseBC, a two-component bacterial adrenergic receptor and global regulator of virulence in Enterobacteriaceae and Pasteurellaceae. *Molecular Oral Microbiology*, 31(5), 379-397. doi:10.1111/omi.12138

Weinstock, G. M. (2012). Genomic approaches to studying the human microbiota. *Nature*, 489(7415), 250-256. doi:10.1038/nature11553

Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., . . . Knight, R. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*, 5, 27. doi:10.1186/s40168-017-0237-y

Weng, R. C. (2017). Dietary fat preference and effects on performance of piglets at weaning. *Asian-Australasian Journal of Animal Sciences*, 30(6), 834-842. doi:10.5713/ajas.16.0499

WHO. (2015). Global Action Plan on Antimicrobial Resistance. Retrieved from http://www.who.int/antimicrobial-resistance/publications/global-action-plan/en/

Willamil, J., Creus, E., Pérez, J. F., Mateu, E., & Martín-Orúe, S. M. (2011). Effect of a microencapsulated feed additive of lactic and formic acid on the prevalence of *Salmonella* in pigs arriving at the abattoir. *Archives of Animal Nutrition*, 65(6), 431-444. doi:10.1080/1745039X.2011.623047

Wilmore, D. W. (1997). Metabolic support of the gastrointestinal tract: Potential gut protection during intensive cytotoxic therapy. *Cancer*, 79(9), 1794-1803. doi:10.1002/(SICI)1097-0142(19970501)79:9<1794::AID-CNCR23>3.0.CO;2-S

Wilson, R. F., & Wilkins, R. J. (1973). Formic acid as a silage additive. *The Journal of Agriculture Science*, 81(1), 117-124. doi:10.1017/S0021859600058391

Woolford, M. K. (1975). Microbiological screening of the straight chain fatty acids (c1-c12) as potential silage additives. *Journal of the Science of Food and Agriculture*, 26(2), 219-228. doi:10.1002/jsfa.2740260213

Xiao, L., Estellé, J., Kiilerich, P., Ramayo-Caldas, Y., Xia, Z., Feng, Q., . . . Wang, J. (2016). A reference gene catalogue of the pig gut microbiome. *Nature Microbiology*, 19, 16161. doi:10.1038/nmicrobiol.2016.161

Xu, J., Chen, X., Yu, S., Su, Y., & Zhu, W. (2016). Effects of early intervention with sodium butyrate on gut microbiota and the expression of inflammatory cytokines in neonatal piglets. *PLoS One*, 11(9), e0162461. doi:10.1371/journal.pone.0162461

Xu, X., Chen, S., Wang, H., Tu, Z., Wang, S., Wang, X., . . . Liu, Y. (2018a). Medium-chain TAG improve intestinal integrity by suppressing toll-like receptor 4, nucleotide-binding oligomerisation domain proteins and necroptosis signalling in weanling piglets challenged with lipopolysaccharide. *The British Journal of Nutrition*, 119(9), 1019-1028. doi:10.1017/S000711451800003X

Xu, Y. T., Liu, L., Long, S. F., Pan, L., & Piao, X. S. (2018b). Effect of organic acids and essential oils on performance, intestinal health and digestive enzyme activities of weaned pigs. *Animal Feed Science and Technology*, 235, 110-119. doi:10.1016/j.anifeedsci.2017.10.012

Xu, J., Li, Y., Yang, Z., Li, C., Liang, H., Wu, Z., & Pu, W. (2018c). Yeast probiotics shape the gut microbiome and improve the health of early-weaned piglets. Frontiers in Microbiology, 9, 2011. doi:10.3389/fmicb.2018.02011

Yang, C., Chowdhury, M. A., Huo, Y., & Gong, J. (2015). Phytogenic compounds as alternatives to in-feed antibiotics: Potentials and challenges in application. *Pathogens*, 4(1), 137-156. doi:10.3390/pathogens4010137

Yang, B., Wang, Y., & Qian, P. Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics*, 17, 135. doi:10.1186/s12859-016-0992-y

Yen, H. C., Lai, W. K., Lin, C. S., & Chiang, S. H. (2015). Medium-chain triglyceride as an alternative of in-feed colistin sulfate to improve growth performance and intestinal microbial environment in newly weaned pigs. *Animal Science Journal*, 86(1), 99-104. doi:10.1111/asj.12248

Zeileis, A., Kleiber, C., & Jackman, S. (2008). Regression models for count data in R. *Journal of Statistical Software*, 27(8). doi:10.18637/jss.v027.i08

Zeitz, J. O., Fennhoff, J., Kluge, H., Stangl, G. I., & Eder, K. (2015). Effects of dietary fats rich in lauric and myristic acid on performance, intestinal morphology, gut microbes, and meat quality in broilers. *Poultry Science*, 94(10), 2404-2413. doi:10.3382/ps/pev191

Zeng, X., Sunkara, L. T., Jiang, W., Bible, M., Carter, S., Ma, X., . . . Zhang, G. (2013). Induction of porcine host defense peptide gene expression by short-chain fatty acids and their analogs. *PLoS One*, 8(8), e72922. doi:10.1371/journal.pone.0072922

Zentek, J., Buchheit-Renko, S., Ferrara, F., Vahjen, Van Kessel, A. G., & Pieper, R. (2011). Nutritional and physiological role ofmedium-chain triglycerides and medium-chain fatty acids in piglets. *Animal Health Research Reviews*, 12(1), 83-93. doi:10.1017/S1466252311000089

Zentek, J., Buchheit-Renko, S., Männer, K., & Vahjen, W. (2012). Intestinal concentrations of free and encapsulated dietary medium-chain fatty acids and effects on gastric microbial ecology and bacterial metabolic products in the digestive tract of piglets. *Archives of Animal Nutrition*, 66(1), 14-26. doi:10.1080/1745039X.2011.644916

Zentek, J., Ferrara, F., Pieper, R., Tedin, L., Meyer, W., & Vahjen, W. (2013). Effects of dietary combinations of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and bacterial metabolites in the digestive tract of weaning piglets. *Journal of Animal Science*, 91(7), 3200-3210. doi:10.2527/jas.2012-5673

Zhang, L., Sato, N. S., Watanabe, K., & Suzuki, T. (2003). Functional genetic selection of the decoding center in *E. coli* 16S rRNA. *Nucleic Acids Symposium Series*, 3, 319-320. doi:10.1093/nass/3.1.319

Zhang, H., Chen, Y., Li, Y., Yang, L., Wang, J., & Wang, T. (2014). Medium-chain TAG attenuate hepatic oxidative damage in intra-uterine growth-retarded weanling piglets by improving the metabolic efficiency of the glutathione redox cycle. *British Journal of Nutrition*, 112(6), 876-885. doi:10.1017/S000711451400155X

Zhang, H., Li, Y., Hou, X., Zhang, L., & Wang, T. (2016). Medium-chain TAG improve energy metabolism and mitochondrial biogenesis in the liver of intra-uterine growth-retarded and normal-birth-weight weanling piglets. *British Journal of Nutrition*, 115(9), 1521-1530. doi:10.1017/S0007114516000404

Zhang, L., Wu, W., Lee, Y. K., Xie, J., & Zhang, H. (2018a). Spatial heterogeneity and co-occurrence of mucosal and luminal microbiome across swine intestinal tract. *Frontiers in Microbiology*, 9, 48. doi:10.3389/fmicb.2018.00048

Zhang, J., Chen, X., Liu, P., Zhao, J., Sun, J., Guan, W., . . . Ma, X. (2018b). Dietary *Clostridium butyricum* induces a phased shift in fecal microbiota structure and increases the acetic acid-producing bacteria in a weaned piglet model. *Journal of Agricultural and Food Chemistry*, 66(20), 5157-5166. doi:10.1021/acs.jafc.8b01253

Zhao, W., Wang, Y., Liu, S., Huang, J., Zhai, Z., He, C., . . . Meng, H. (2015). The dynamic distribution of porcine microbiota across different ages and gastrointestinal tract segments. *PLoS One*, 10(2), e0117441. doi:10.1371/journal.pone.0117441

Zhou, M., Guo, Z., Yang, Y., Duan, Q., Zhang, Q., Yao, F., . . . Zhu, G. (2014). Flagellin and F4 fimbriae have opposite effects on biofilm formation and quorum sensing in F4ac+ enterotoxigenic *Escherichia coli*. *Veterinary Microbiology*, 168(1), 148-153. doi:10.1016/j.vetmic.2013.10.014

Zivkovic, A. M., Lewis, Z. T., German, J. B., & Mills, D. A. (2013). Establishment of a milk-oriented microbiota (MOM) in early life: How babies meet their MOMs. *Functional Food Reviews*, 5(1), 3-12. doi:10.2310/6180.2009.00035

Appendix A

Short- and/or medium-chain fatty acids effects on performance and coliform counts

Works evaluating average daily gain (ADG) corresponding to Figure 2.4:

Barba-Vidal et al. (2017a)
Bhandari et al. (2008)
Biagi et al. (2007)
Bosi et al. (2007)
Canibe et al. (2001)
Canibe et al. (2005)
Creus et al. (2007)
De Smet et al. (2016)
Devi et al. (2014)
Dierick et al. (2002a)
Dierick et al. (2004)
Eisemann & van Heugten (2007)
Ettle et al. (2004)
Fang et al. (2014)
Feng et al. (2018)
Han et al. (2011)
Hanczakowska et al. (2010)
Hanczakowska et al. (2011)
Hanczakowska et al. (2013)
Hanczakowska et al. (2016a)
Hanczakowska et al. (2016b)
He et al. (2016)
Hernández & Pluske (2008)
Hong et al. (2012)
Htoo & Morales (2012)
Huang et al. (2015)
Kil et al. (2006)
Lai et al. (2014)
Li et al. (2008)
Li et al. (2015)
Long et al. (2018)
Lu et al. (2008)
Lu et al. (2012)
Luise et al. (2017)
Mallo et al. (2012)
Manzanilla et al. (2004)
Manzanilla et al. (2006)
Marounek et al. (2004)
Mazzoni et al. (2008)
Michiels et al. (2012)
Miller, et al. (2016)
Øverland et al. (2000)
Øverland et al. (2008)
Partanen et al. (2002a)
Partanen et al. (2007)
Piva et al. (2002)
Poeikhampha & Bunchasak (2011)
Soler et al. (2018)
Stensland et al. (2015)
Tsiloyiannis et al. (2001)
Upadhaya et al. (2014)
Upadhaya et al. (2016)
Upadhaya et al. (2018)
Walia et al. (2016)
Weber & Kerr (2008)
Weber et al. (2014)
Yen HC, et al. (2015)
Zentek et al. (2012)
Zentek et al. (2013)
Zhang et al. (2014)

Works evaluating gain-to-feed ratio (GF) corresponding to Figure 2.5:

Barba-Vidal et al. (2017a)
Bhandari et al. (2008)
Biagi et al. (2007)
Bosi et al. (2007)
Canibe et al. (2001)
Canibe et al. (2005)
Creus et al. (2007)
Devi et al. (2014)
Dierick et al. (2002a)
Dierick et al. (2004)
Eisemann & van Heugten (2007)
Ettle et al. (2004)
Fang et al. (2014)
Feng et al. (2018)

Han et al. (2011)

Hanczakowska et al. (2010)

Hanczakowska et al. (2011)

Hanczakowska et al. (2011)

Hanczakowska et al. (2013)

Hanczakowska et al. (2016b)

Hong et al. (2012)

Htoo & Morales (2012)

Huang et al. (2015)

Kil et al. (2006)

Lai et al. (2014)

Li et al. (2008)

Li et al. (2015)

Long et al. (2018)

Lu et al. (2008)

Luise et al. (2017)

Mallo et al. (2012)

Manzanilla et al. (2004)

Manzanilla et al. (2006)

Mazzoni et al. (2008)

Miller et al. (2016)

Øverland et al. (2000)

Øverland et al. (2008)

Partanen et al. (2002a)

Partanen et al. (2007)

Piva et al. (2002)

Poeikhampha & Bunchasak (2011)

Tsiloyiannis et al. (2001)

Upadhaya et al. (2014)

Upadhaya et al. (2016)

Upadhaya et al. (2018)

Walia et al. (2016)

Weber & Kerr (2008)

Weber et al. (2014)

Yen et al. (2015)

Zentek et al. (2013)

Zhang et al. (2014)

Works evaluating average daily feed intake (ADFI) corresponding to Figure 2.6:

Barba-Vidal et al. (2017a)

Bhandari et al. (2008)

Biagi et al. (2007)

Bosi et al. (2007)

Canibe et al. (2001)

Canibe et al. (2005)

Creus et al. (2007)

Devi et al. (2014)

Dierick et al. (2002a)

Eisemann & van Heugten (2007)

Ettle et al. (2004)

Fang et al. (2014)

Feng et al. (2018)

Han et al. (2011)

Hanczakowska et al. (2016b)

Hong et al. (2012)

Htoo & Morales (2012)

Huang et al. (2015)

Kil et al. (2006)

Lai et al. (2014)

Li et al. (2008)

Li et al. (2015)

Long et al. (2018)

Lu et al. (2008)

Luise et al. (2017)

Mallo et al. (2012)

Manzanilla et al. (2004)

Manzanilla et al. (2006)

Mazzoni et al. (2008)

Miller et al. (2016)

Øverland et al. (2000)

Øverland et al. (2008)

Partanen et al. (2007)

Piva et al. (2002)

Poeikhampha & Bunchasak (2011)

Soler et al. (2018)

Stensland et al. (2015)

Tsiloyiannis et al. (2001)

Upadhaya et al. (2014)

Upadhaya et al. (2016)

Upadhaya et al. (2018)

Walia et al. (2016)

Weber & Kerr (2008)

Weber et al. (2014)

Willamil et al. (2011)

Yen et al. (2015)

Zentek et al. (2012)

Zentek et al. (2013)

Zhang et al. (2014)

Works evaluating coliform counts in digesta corresponding to Figure 2.8:

Bhandari et al. (2008)

Canibe et al. (2001)

Canibe et al. (2005)

Devi et al. (2014)

Dierick et al. (2004)

Franco et al. (2005)

Hanczakowska et al. (2010)

Hanczakowska et al. (2011)

Hanczakowska et al. (2016b)

Lai et al. (2014)

Li et al. (2018)

Long et al. (2018)

Lu et al. (2008)

Manzanilla et al. (2004)

Manzanilla et al. (2006)

Øverland et al. (2008)

Upadhaya et al. (2016)

Upadhaya et al. (2018)

Yen et al. (2015)

Zentek et al. (2012)

Zentek et al. (2013)

Appendix B

Gastrointestinal microbiota by high-throughput sequencing (HTS) of 16S rRNA gene

Works profiling faecal microbiota over pig's life corresponding to Figure 2.13:

Bian et al. (2016)
Chen et al. (2017)
Frese et al. (2015)
Han et al. (2018)
Kim & Isaacson (2015)
Kim et al. (2011)
Lamendella et al. 2011
Looft et al. (2014b)
Mach et al. (2015)
Niu et al. (2015)
Pajarillo et al. (2014)
Pollock et al. (2018)
Poole et al. (2013)
Poulsen et al. (2018)
Slifierz et al. (2013)
Solano-Aguilar et al. (2018)
Soler et al. (2018)
Xu et al. (2018b)
Zhang et al. (2018b)
Zhao et al. (2015)

Works evaluating gastrointestinal lumen microbiota corresponding to Figure 2.14:

Bin et al. (2018)
De Rodas et al. (2018)
Li et al. (2018)
Looft et al. (2014a)
Luise et al. (2017)
Motta et al. (2018)
Mu et al. (2017)
Newman et al 2018
Poulsen et al. (2018)
Xu et al. (2016)
Zhang et al. (2018a)
Zhao et al. (2015)

Works evaluating gastrointestinal mucosa microbiota corresponding to Figure 2.15:

Borewicz et al. (2015)
De Rodas et al. (2018)
Looft et al. (2014a)
Motta et al. (2018)
Mu et al. (2017)
Zhang et al. (2018a)